

**TELOMERE BIOLOGY IN THE FRESHWATER PLANARIAN  
SCHMIDTEA MEDITERRANEA**

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*Yet a little while, and the world seeth me no  
more; but ye see me: because I live, ye shall  
live also.*

(John 14:19)

## Abstract

Freshwater planarian *Schmidtea mediterranea* is an emerging model for studying *in vivo* gene functions and regulation in native cell niches. The obligate asexual strain of this species reproduces by fission, in which succession of soma occurs without passing through the germline. To achieve this somatic immortality the somatic stem cells need to overcome the end replication problem. Therefore it can be hypothesised that somatic telomere maintenance in asexual *S. mediterranea* must possess a germ-like property, with which age-related erosions can be adequately repaired. In this PhD project, the telomere repeat unit in *S. mediterranea* was confirmed to be the vertebrate-like TTAGGG. Attrition of whole body telomere length was found in ageing sexual worms and also in asexual worms which had not gone through recent fission events. Opposite telomere length dynamics were observed in regenerated samples of the two strains, with erosion in the sexuals and reset in the asexuals. The telomere maintenance was found to increase during regeneration in both strains, with a higher level of increase in asexual worms. A homolog of the telomerase reverse transcriptase subunit, *Smed\_Tert*, was identified and characterised in this organism. High level of *Smed\_Tert* expression was seen in germ cells in mature sexual worms and adult stem cells in asexual worms. Knockdown of *Smed\_Tert* expression by RNA interference caused progressive telomere erosion, however effects on cell proliferation and viability have not been observed in knockdown samples. Four alternate splice isoforms of *Smed\_Tert* were identified. The enhanced telomerase activity during regeneration correlates with a proportional increase in the full-length isoform and a decrease in isoforms with a truncated TRBD domain, suggesting a dominant negative regulation of telomerase by alternative splicing. Significant increase in the expression of the full-length isoform was seen in regenerating asexual samples but not in sexual

strains, which correlates with their telomere length dynamics. It is hoped that the comparative studies between the sexual and asexual strains can improve our understanding of how soma can evolve to become an effective inheritable unit.

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## **Abbreviations**

### ***Experimental techniques***

CHEF – Clamped Homogeneous Electrical Field

FACS – Fluorescence Activated Cell Sorting

FIGE – Field Inversion Gel Electrophoresis

qRT-PCR – Quantitative Reverse Transcription Polymerase Chain Reaction

RACE – Rapid Amplification of cDNA Ends

RNAi – RNA Interference

STELA – Single Telomere Length Analysis

TRAP – Telomere Repeat Amplification Protocol

TRF – Terminal Restriction Fragment

### ***General biological terms***

cDNA – Complementary DNA

gDNA – Genomic DNA

mRNA – Messenger RNA

NMD – Nonsense Mediated Decay

ROS – Reactive Oxygen Species

UTR – Untranslated Region

### ***Chemicals and reagents***

BSA – Bovine Serum Albumin

DAPI – 4',6-diamidino-2-phenylindole

DMSO – Dimethyl sulfoxide

DTT – Dithiothreitol

EtOH – Ethanol

MetOH – Methanol

PBS – Phosphate Buffered Saline

PFA – Paraformaldehyde

PVA – Polyvinyl Alcohol

SDS – Sodium Dodecyl Sulphate

TEA – Triethylamine

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# CHAPTER 1: INTRODUCTION

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## 1.1 Telomeres, Cellular Senescence, and Life Span

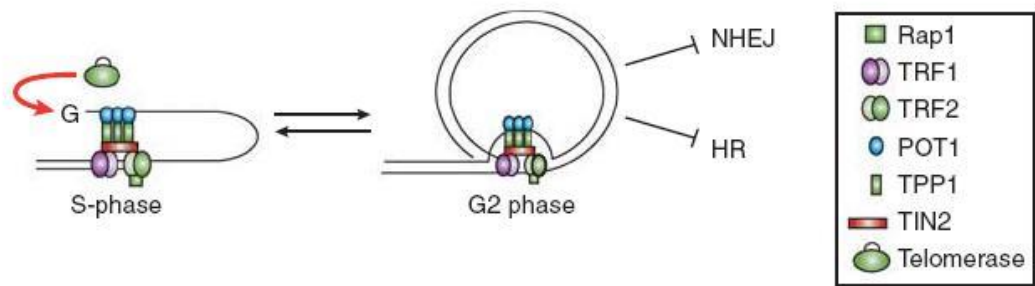
### 1.1.1 The General Structure of Telomeres

The telomere is a nucleoprotein complex at the end of eukaryotic chromosomes. It consists of a tandem G-rich DNA repeat which varies in repeating sequence and length between species. The very end of telomeres is characterised by a 3' single stranded overhang required to form the unique secondary structure of this chromosome region. Electron microscopy of cross-linked chromosomes from human cells has observed the invasion of the 3' overhang into an internal telomeric site, forming a 75-200 bp displacement loop (D-loop) at the site of invasion, and a closed circular structure between the chromosome end and the invasion site termed the t-loop (Griffith et al., 1999). The t-loop has been found in a wide variety of eukaryotic organisms such as human, mouse (Griffith et al., 1999), the hypotrichous ciliate *Oxytricha fallax* (Murti and Prescott, 1999), the flagellate protozoan *Trypanosoma brucei* (Muñoz-Jordán et al., 2001), and the plant *Pisum sativum* (Cesare et al., 2003); and is therefore thought to be the most conserved feature of eukaryotic chromosome ends (reviewed in (de Lange, 2004)). In humans, telomeric DNA is bound by double stranded repeat binding proteins TRF1 (telomeric repeat binding factor 1) (Zhong et al., 1992, Chong et al., 1995) and TRF2 (Bilaud et al., 1997), and a single stranded telomere binding protein POT1 (protection of telomeres 1) (Baumann and Cech, 2001) (reviewed in (De Boeck et al., 2009)). Both TRF1 and TRF2 function as homodimers; they share a similar

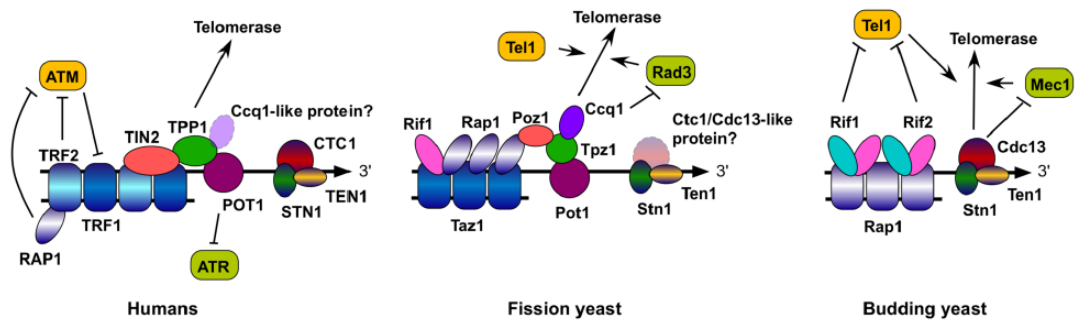
protein structure, with a C-terminal Myb domain which confers their specificity to the telomeric DNA. The major difference between the two proteins resides at their N-terminal end, which is acidic in TRF1 and basic in TRF2 (Broccoli et al., 1997). TRF1 has the ability of induce DNA bending and looping, which assists the formation of the t-loop (Griffith et al., 1999, Bianchi et al., 1997), while TRF2 facilitates the 3' overhang invasion and stabilises the D-loop (Griffith et al., 1999, Stansel et al., 2001). Both proteins negatively regulate telomerase function by sequestration of the 3' overhang in the t-loop (Smogorzewska et al., 2000). Overexpression of wild-type TRF1 causes gradual telomere shortening, while expression of a dominant negative allele of TRF1 increases telomere length (van Steensel and de Lange, 1997). In contrast, overexpression of TRF2 was found to transiently decrease telomere length (Smogorzewska et al., 2000), and inhibition or deletion of TRF2 results in loss of 3' overhang and an ATM-dependent activation of DNA damage signals, which leads to genome instability, chromosome end-to-end fusion, cellular senescence and cell death (van Steensel et al., 1998, Denchi and De Lange, 2007). POT1 was demonstrated to control the access of telomerase and 5' end resectioning enzymes which combinatorially regulate the length of 3' overhangs. Furthermore, POT1 inhibits an ATR-mediated DNA damage response and prevents telomere dysfunctions (Denchi and De Lange, 2007) (reviewed in more detail in Chapter 6).

These telomeric sequence binding proteins are additionally bound by RAP1, TIN2, and TPP1 to form a functional telomere complex called shelterin (see Figure 1.1 and 1.2). RAP1 (repressor activator protein 1) is recruited to the telomere by binding to TRF2, its overexpression causes telomere elongation in a telomerase-dependent manner (Li and De Lange, 2003),

however its inhibition by shRNA or dominant negative allele expression also results in telomere extension (O'Connor et al., 2004). Additionally, RAP1 interacts with proteins in DNA damage response pathways, including RAD50, MRE11, KU70/86 and PARP1, indicating a possible role of these proteins in telomere length dynamics (O'Connor et al., 2004). TIN2 (TRF1 interacting nuclear protein 2) simultaneously binds to TRF1 (Kim et al., 1999), TRF2, and TPP1 (Houghtaling et al., 2004) in the complex. It was shown to protect TRF1 from inhibition by tankyrase 1, and regulates telomere length by controlling the number of TRF1 molecules accumulated at the telomere (Ye and de Lange, 2004). TPP1 (TIN2 and POT1 interacting protein 1) bridges POT1 with the TIN2/TRF1/TRF2 complex, it was initially shown to inhibit telomerase access by pairing with POT1 (Liu et al., 2004a, Ye et al., 2004), recently this heterodimeric pair was found to also recruit telomerase by associating with the holoenzyme by the oligonucleotide/oligosaccharide binding (OB) fold in TPP1 (Xin et al., 2007a). The pair was also demonstrated to enhance telomerase processivity by slowing down the dissociation of telomerase from the 3' overhang and increasing its translocation along the substrate (Wang et al., 2007a, Latrick and Cech, 2010).



**Figure 1.1 – Schematic model of human shelterin in S-phase and G2 phase.** Only the shelterin complex at the very end of the telomere is shown, binding of the same complex along the internal double stranded telomere is left out from the illustration. (Adapted from (Deng and Chang, 2007))



**Figure 1.2 – Evolutionarily conserved telomere binding proteins (shelterin and CST complex).** The similarities and differences in their functions between human, fission yeast, and budding yeast are illustrated. Evolutionarily conserved proteins are shown in the same colours. (Adapted from Subramanian and Nakamura, 2010)

In summary, the shelterin complex acts as a protective cap which controls telomere maintenance and prevents chromosome ends from being recognised as double strand breaks which can trigger the ATM or ATR-mediated DNA damage response pathways. Additionally, it shields chromosome ends from enzymatic degradation, non-homologous end joining (NHEJ), and homologous recombination which lead to rapid

telomere length alteration, genome instability, cell cycle arrest, and apoptosis.

Comparative studies between organisms have revealed diverged structures of shelterin complexes and rapidly evolving functions of telomere related proteins (reviewed in (Subramanian and Nakamura, 2010)). Fission yeast *Schizosaccharomyces pombe* has only one double stranded telomere binding protein, Taz1, which is evolutionarily related to the human TRF1/TRF2 (Cooper et al., 1997). In contrast to the uncapped telomere phenotypes in human cells without TRF2, deletion of *Taz1* in *S. pombe* induces NHEJ without significant activation of checkpoint proteins, and cells remain proliferative and viable with an increase in telomere length (Cooper et al., 1997, Miller and Cooper, 2003). Rif1 (homolog of Rap1 interacting factor 1 in *S. cerevisiae*), a general DNA damage response protein which does not bind to normal undamaged telomeres in humans, was shown to associate directly with Taz1 in fission yeast telomeres and possess length regulation function (Kano and Ishikawa, 2001). Deletion of Pot1 in fission yeast produces severe chromosome fusions and cell death (Baumann and Cech, 2001), while inhibition of the gene in human cells results in a mild (2-fold) increase in telomere fusions, in contrast to 10-100 fold after TRF2 inhibition (Hockemeyer et al., 2005), indicating a greater role of the single stranded telomere binding protein in telomere capping in *S. pombe*; alternatively, other proteins at this region may buffer the effect of POT1 knockdown in humans.

TRF1/TRF2-related proteins are absent in budding yeast *Saccharomyces cerevisiae*. Rap1 binds directly to the double stranded telomeric DNA (Conrad et al., 1990), recruiting Rap1-interacting factors Rif1 and Rif2 for

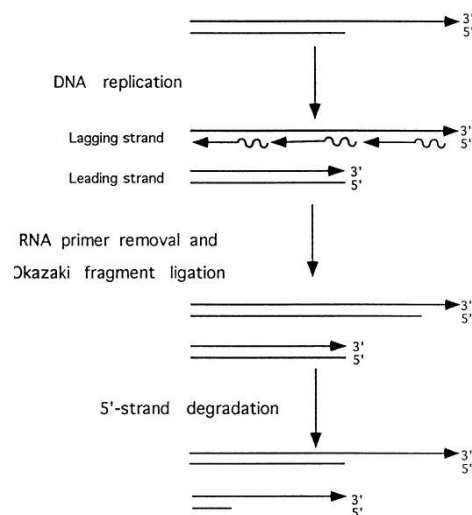


telomere length regulation (Hardy et al., 1992, Wotton and Shore, 1997) and prevention of Tel1 (ATM homolog)-mediated DNA damage responses (Hirano et al., 2009). Similar to Taz1 in *S. pombe*, conditional repression of Rap1 in budding yeast induces chromosome fusions by the NHEJ pathway (Pardo and Marcand, 2005). A homolog of Pot1 is absent in *S. cerevisiae* at the sequence level, instead Cdc13, which shares structural homology with Pot1, binds to the single stranded telomere (Garvik et al., 1995), together with another two OB fold-containing proteins Stn1 and Ten1 which are also specific to single stranded telomeric sequence, forming a CST (Cdc13-Stn1-Ten1) complex which caps the 3' overhang (Grandin et al., 1997, Grandin et al., 2001). The CST complex is the telomere specific analogue of the heterotrimeric replication protein A (RPA) complex (Gao et al., 2007, Gelinias et al., 2009, Sun et al., 2009), it shields telomeres from Rad9-mediated checkpoint activation, inhibits NHEJ, controls C-strand resections, and regulates telomerase access to the 3' overhangs (Grandin et al., 2001). Stn1 and Ten1 are also present in fission yeast, their deletion phenotypes are identical to Pot1 deletion which includes elongation of cells, cell death, and circularisation of chromosomes. However in contrast with the interaction between Cdc13-Stn1-Ten1 in *S. cerevisiae*, yeast two-hybrid experiments detect interaction between Stn1-Ten1 and Pot1-Pot1 but not between Stn1-Pot1 and Ten1-Pot1 in *S. pombe*, suggesting two independent single strand telomere binding complexes which are both required to prevent the DNA damage signal (Martín et al., 2007). The plant and mammalian counterparts of this complex have been identified in *Arabidopsis thaliana*, humans, and mice; the complex consists of CTC1, STN1, and TEN1, their functions are similar to the yeast homologs in telomere capping (Song et al., 2008, Surovtseva et al., 2009, Miyake et al., 2009). Mammalian CTC1 differs from the budding yeast Cdc13 in that

it does not bind specifically to telomere sequence (Miyake et al., 2009), and may function more generally as a cofactor of DNA polymerase- $\alpha$  primase to regulate DNA replication (Casteel et al., 2009). Nonetheless, inhibition of CTC1 in human cells causes DNA damage signals, defective chromosome segregations, sporadic loss of telomeres, and accumulation of 3' overhangs, indicating its essential role in telomere integrity (Surovtseva et al., 2009). The presence of the CST complex across eukaryotic species suggests that it may be the most conserved mechanism for telomere capping (reviewed in (Subramanian and Nakamura, 2010)).

### **1.1.2 Telomere shortening and cellular senescence**

As a result of the replication of linear-structured eukaryotic chromosomes, the telomere erodes every time a cell divides (Olovnikov, 1971, Watson, 1972, Olovnikov, 1973). At the final stage of lagging strand synthesis, the RNA primer at the most 3' end of the lagging strand is degraded and the resulting lesion (generally 5 to 15 nucleotides in length) cannot be repaired by canonical DNA polymerase and ligase. The 5' end of the newly synthesised strands, together with the 5' end of the original template strands are further degraded by a 5'-3' exonuclease activity (Makarov et al., 1997), resulting in 3' overhangs that are maintained at a constant length and a decrease in the total telomere length (see Figure 1.3). Progressive loss of average telomere length with age has been observed *in vivo* and *in vitro* in mammals (Kveiborg et al., 1999, Vaziri et al., 1994, Fiset and Chabot, 2001). It was discovered that in some human epithelial cell types, telomere length decreases by 50-100 bp per population doubling, this accounts for 2-4 kb over the life time of a person (reviewed in (Deng and Chang, 2007)).



**Figure 1.3 – The degradation model of telomere shortening.** Following complementary strand synthesis, the 5' end of each strand is degraded by an exonuclease activity, resulting in a fixed length of 3' overhang. (Adapted from (Makarov et al., 1997))

In addition to the gradual telomere loss due to the end replication problem, abrupt loss of telomeres can also occur. This sudden attrition can arise by oxidative damage-related single-strand breaks causing replication fork stalling and/or recombination (von Zglinicki et al., 1995, Sitte et al., 1998, Passos et al., 2007) unequal sister chromatid exchange (Baird et al., 2003, Baird, 2009), and intra-chromosomal telomeric recombination which results in formation of an extrachromosomal telomere loop and distal chromosome end deletion (Rubelj and Vondracek, 1999, Wang et al., 2004). However these events occurred in *ex vivo* cell cultures, some with artificially induced stresses. *In vivo* observations indirectly suggest that intense and chronic psychological stress is linked to high oxidative stress, low telomerase activity, short telomere length, and early onset of ageing-related diseases (Epel et al., 2004, Epel et al., 2006, Simon et al., 2006). The abundance of abrupt telomere length changes *in vivo*, where cells are in their native environments, the significance of these events in the stem cell pool, and their contributions to whole body ageing, require further experimental confirmation.

When telomere length reaches a critical point, the structure of the protective cap complex cannot be maintained. This dysfunctional telomere is recognised as a double strand break, and is detected by sensor proteins such as MRE11, NBS1, and RAD50. These proteins activate a signal transduction pathway which involves the protein kinases ATM/ATR, followed by CHK1, CHK2, and p53, which triggers a tumour suppressing mechanism involving apoptosis or long term cell cycle arrest via the p21 pathway (ie. cellular senescence) (reviewed in (Deng and Chang, 2007)). Additionally, the p16<sup>INK4a</sup>/pRb pathway has a similar effect on the induction of cellular senescence in humans (Shay et al., 1991), but not in mice (Yi et al., 2000).

It was observed in human fibroblasts that telomere-mediated cellular senescence involves two phases, mortality stages 1 and 2 (M1 and M2) (reviewed in (Yi et al., 2000)). M1 corresponds to the stage in which telomere(s) reaches a certain length that is detected by the DNA damage checkpoint as a double strand break which requires repairing, this results in a cell cycle arrest mediated by the p53 and/or p16<sup>INK4a</sup>/pRb pathway. Mutations in these pathways, or expression of viral oncogenes can result in cells escaping from the cycle arrest and resuming division despite their short telomere. This will lead to M2 (sometimes termed the crisis stage), in which terminally short telomere(s) induces NHEJ, which leads to breakage-fusion bridge, gross chromosomal rearrangements, and consequently apoptosis or necrosis.

Cellular senescence may act as a suppressive mechanism to avoid rapid proliferation of tumour cells, however it was seen in human fibroblasts that senescent cells secrete pro-inflammatory factors which promote malignant

transformation and propagation of neighbouring epithelial cells (Krtolica et al., 2001). This recalls the evolutionary theory of antagonistic pleiotropy (Williams, 1957) in a way that the senescence mechanism favours individual fitness in early life by suppressing cancer development, while having an unselected chronic effect on promoting tumorigenesis as senescent cells accumulate in aged organisms (Krtolica et al., 2001).

### **1.1.3 Telomere shortening, whole body ageing, and life span**

Cells without adequate telomere maintaining mechanisms have a finite capacity for replication. This cell division limit, originally documented in human normal fibroblast culture (Hayflick and Moorhead, 1961), is termed the Hayflick limit. Somatic stem cells in mortal organisms have characteristics of weak/incomplete telomere maintenance, and it can be hypothesised that the whole body ageing of an organism results from the gradual increase in the proportion of its stem cells, responsible for the homeostatic maintenance of its reproduction, defence, and repair functions, reaching their Hayflick limit and becoming senescent (reviewed in (Weinstein and Ciszek, 2002)). Ectopic activation of a telomere maintenance enzyme, telomerase, is able to delay cellular senescence in human cell lines (Bodnar et al., 1998). The correlation between telomere erosion with advancing age, both in whole body and in individual cell types, in addition to the anti-senescent effect of enhancing telomere maintenance, suggests an essential role for telomere length in determining the Hayflick limit of somatic stem cells, and the possible consequential effect on the life span of the organism.

In addition to the Hayflick limit theory, a lab strain of nematode *Caenorhabditis elegans* with long telomeric DNA generated by transgenically overexpressed HRP-1 protein has an increased life span (Joeng et al., 2004). HRP-1 is a homolog of human RNP A1, which was shown to bind specifically to single strand telomeres and regulate telomere length, possibly by recruiting and stimulating telomerase and DNA polymerase  $\alpha$  (LaBranche et al., 1998, Fiset and Chabot, 2001). The extended life-span in HRP-1 overexpressed samples is dependent on the activity of a Fork head transcription factor DAF-16, which has previously been found to affect the life span of the organism via insulin receptor-like signalling from the reproductive system (Ogg et al., 1997, Hsin and Kenyon, 1999), however it was found that the effect of long telomeres is independent of the cycling of the germ stem cells (Joeng et al., 2004). This suggests that telomere length can also affect the longevity of an organism independently of the replicative capacity of the stem cells.

However, variations in the telomere length between different species do not correlate with the difference in their life spans. Comparison between human and mouse has revealed that lab mice have on average a much longer telomere (Kipling and Cooke, 1990), while having a much shorter life span. It was also found that cultured mouse embryonic fibroblasts enter a transient replicative senescence stage after 10-15 population doublings, despite the presence of intact telomeres and telomerase activity (Blasco et al., 1997). These findings suggest a difference in the limits of telomere length as a Hayflick limit determinant between human and mouse, and possibly between other long-lived and short-lived organisms.

Despite the small but significant negative correlation of telomere length with age, currently published data have shown that the mean terminal restriction fragment (TRF) lengths in older human individuals (usually above 7 kb) are still significantly longer than those in senescent cells (around 4 kb) (reviewed in (Baird and Kipling, 2004)). Moreover, a study looking at the *in vitro* replicative life span of fibroblasts explanted from donors of different ages has shown no significant difference between young and old cells (Cristofalo et al., 1998). With conventional TRF technique it is difficult to pin-point the length of telomere which triggers senescence, since it lacks the resolution for individual chromosome ends. Furthermore, samples used in the TRF study were obtained from native tissues, of which the mean TRF lengths represent the majority of cells which are more likely to be descendants of healthy proliferative stem cells instead of senescent cells. As seen in the hematopoietic compartment, a pool of quiescent stem cells exists, possibly in order to replace the rapidly-dividing stem cells when they senesce (Fleming et al., 2008). It can therefore be hypothesised that the whole body ageing is caused by the senescence of a relatively small but rapidly dividing fraction of stem cells, of which the telomere lengths are not visible and neglected in conventional TRF analysis. The factors secreted by these cells, such as extracellular matrix-degrading enzymes, cytokines, and growth factors, compromise tissue functions and promote malignant transformation of neighbouring cells, and ultimately trigger tissue dysfunctions and cancers (Krtolica et al., 2001). Alternatively, telomere-independent factors such as oxidative stress may trigger cellular senescence and whole body ageing prior to telomere attrition in some individuals.

#### 1.1.4 Telomere maintenance mechanisms

The telomeres of actively dividing cells are predominantly maintained by the activity of enzyme telomerase. Telomerase is a ribonucleoprotein complex which recognises and binds to the 3' overhang of the telomere during the S-phase of the cell cycle (Greider and Blackburn, 1985, Zhu et al., 1996). It contains an integral RNA subunit (TERC) with a telomere-binding region which is complementary to the 3' telomere end, and an adjacent region which acts as a template for the telomeric repeat synthesis (Greider and Blackburn, 1989). The reverse transcriptase subunit (TERT) catalyses the addition of new bases to 3' overhangs according to the RNA template, resulting in elongation of the G-strand telomeres (Lingner et al., 1997, Counter et al., 1997, Harrington et al., 1997). The complementary strand is then lengthened by DNA polymerase  $\alpha$ -primase, using the 3' overhang as the template (Diede and Gottschling, 1999, Nozawa et al., 2000). TERC and TERT are the two components required to reconstitute telomerase activity *in vitro* (Weinrich et al., 1997), while telomerase *in vivo* is bound with various factors important for the ribonucleoprotein stability and recruitment (reviewed in (Collins, 2006)), forming a large holoenzyme containing a dimer or multimer of TERC and TERT (Prescott and Blackburn, 1997, Beattie et al., 2001). In contrast to canonical reverse transcriptase enzymes, telomerase has a unique ability to synthesise multiple units of telomeric repeat with a short template, which is usually 1.5 copies of the unit (Greider, 1991). This feature is achieved by translocating the catalytic site and RNA template along the strand, adding telomeric sequences repetitively (Peng et al., 2001). Telomerase can be found across the eukaryotes, from diplomonads (Malik et al., 2000) to human. Recruitment of telomerase to telomere ends by the shelterin complex and telomerase component was demonstrated in budding yeast



by the interaction between Est1, which binds Tlc1 (yeast telomerase RNA subunit), and Cdc13 (Evans and Lundblad, 1999, Qi and Zakian, 2000, Bianchi et al., 2004). Two of the human Est1 homologs (EST1A and B) were also shown to be telomerase components. Both proteins bind to TERT independent of TERC, and EST1A shows high affinity to single stranded telomeric sequence (Snow et al., 2003). Overexpression of EST1A induces telomere uncapping (Reichenbach et al., 2003), suggesting its role in opening the t-loop structure which allows access of telomerase.

TERC between organisms are highly diverged at the primary sequence level (reviewed in (Nugent and Lundblad, 1998)), despite the conserved functions of these RNAs. Comparison of TERCs from distantly related ciliates has suggested a conserved secondary structure between species (Romero and Blackburn, 1991). This is supported by a phylogenetic study which discovered the consensus secondary structures in vertebrate TERCs, these include the pseudoknot domain, the CR4-CR5 domain, the H/ACA box, and the CR7 domain (Chen et al., 2000). Multi-species alignment of TERT protein has revealed a conserved general protein structure (see Figure 1.4, reviewed in (Kelleher et al., 2002)), with telomerase specific domains at both N and C-terminals, surrounding a reverse transcriptase (RT) catalytic domain which contains conserved sequence motifs (Motif 1, 2, A, B', C, D, and E) with viral reverse transcriptases and retrotransposons (Nakamura et al., 1997). The N-terminal region (TEN) consists of two telomerase-specific sequences motifs (Motif GQ and DAT), it possesses affinity to single stranded DNA and is essential for the activity and processivity of telomerase (Xia et al., 2000, Armbruster et al., 2001, Jacobs et al., 2006, Romi et al., 2007, Lue, 2005), the sequence specificity of this protein-DNA interaction varies between organisms (Xia et al., 2000,

Sealey et al., 2009). Between the TEN and RT domain is the telomerase RNA binding domain (TRBD) which includes conserved Motif CP, QFP, and T (Friedman and Cech, 1999, Xia et al., 2000). This domain interacts with TERC and is essential for the functional multimerisation of telomerase components and ribonucleoprotein assembly (Moriarty et al., 2002, Bosoy et al., 2003). Differed from RTs of retrovirus and retrotransposons, a unique feature of TERT RT domain is the insertion of a conserved IFD motif between Motif A and B', which is required for DNA primer recognition and processive addition of telomeric repeat (Lue et al., 2003). The C-terminus of the protein was also found to be essential for processivity (Peng et al., 2001, Hossain et al., 2002, Huard et al., 2003). While having no significant sequence homology to retroviral RTs, this region of TERT may also form a "thumb" domain which is important for template/primer binding in its retroviral relatives (Peng et al., 2001, Hossain et al., 2002).



**Figure 1.4 – A general TERT protein structure as represented by hTERT.** Multi-species conserved sequence motives are illustrated as boxes with unique colours. TRBD: Telomerase RNA Binding Domain.

Telomerase activity is absent in most normal somatic human tissues and mortal cell lines (LaBranche et al., 1998, Wright et al., 1996), where TERC is lowly expressed and TERT is either low or not detectable (Nakamura et al., 1997, Meyerson et al., 1997). While in telomerase-positive cancer cell lines, expression of both components is highly up-regulated (Nakamura et

al., 1997, Meyerson et al., 1997, Yi et al., 1999) which correlates with the requirement for counteracting the end replication problem in these rapidly dividing cells. Similarly, telomerase activity can be detected in some proliferative normal cell types; high level of activity is present in male germ cells and activated lymphocytes (Wright et al., 1996, Weng et al., 1996), while low levels can be seen in peripheral blood lymphocytes and hematopoietic progenitor cells (Hiyama et al., 1995). Malfunctioning of this holoenzyme results in rapid telomere erosion and genome instability, which lead to depletion of proliferative cells due to replicative senescence or apoptosis (Lundblad and Szostak, 1989, Lee et al., 1998). While expression of exogenous TERT in somatic cell lines was found to restore the proliferative capacity and allow the cells to divide beyond their usual population doubling limit (Bodnar et al., 1998). It is estimated that 90%-95% of human cancer cells are telomerase positive (reviewed in (Liu et al., 2004b)), although this percentage is highly variable in different cancer types (such as 75%-95% in breast carcinoma, and 11%-45% in fibroadenoma) (reviewed in (Meyerson, 2000) ).

Several alternative mechanisms for lengthening telomere (termed ALTs) were found in some telomerase-negative yeast strains, human cancer cells, and immortalised cell lines (reviewed in (Henson et al., 2002)). General characteristics of ALT cell populations include presence of ALT-associated promyelocytic leukaemia (PML) bodies, and a highly heterogeneous distribution of telomere lengths ranging from 3kb to 50kb, as measured by TRF analysis (Bryan et al., 1995). Fluorescent in-situ hybridisation (FISH) on metaphase chromosomes has shown that this heterogeneity is present within individual cells, in which some chromosome ends are barely detectable, while some have very strong signals (Perrem et al., 2001).

These data suggests that homologous recombination events have occurred between chromosome ends, with which critically short ends obtain a section of telomeric sequence from longer ends. This mechanism results in rapid changes in telomere lengths, in contrast with the ends maintained by telomerase which are relatively constant (reviewed in (Henson et al., 2002)). Another possible ALT mechanism involves the single-stranded 3' overhang invading a homologous double-stranded telomeric repeat array, followed by the synthesis of new telomeric sequences at the site of invasion (proposed by (Henson et al., 2002)). This is similar to the formation of t-loop at the G2 phase of normal cells, except that the replication event in normal cells is inhibited, possibly by the presence of POT1 proteins (Baumann and Cech, 2001). In addition to the same chromosome end, the invasion can also occur in different telomeres or extrachromosomal telomeric repeat DNA (ECTR) (Regev et al., 1998, Ogino et al., 1998). It is hypothesised that the ECTR sequences present in ALT cells can elongate telomeres by end-joining, homologous recombination, or act as templates for telomere synthesis (Henson et al., 2002). The ALT-associated PML body (APB) contains proteins that are mostly involved in DNA break repair via recombination, such as RAD52, RAD51, and the MRE11/RAD50/NBS1 complex. As shown by yeast homologs, Rad51 is required for the type I telomerase-null survival pathway, which involves amplification of subtelomeric Y' elements; while Rad50 is required for the type II pathway which elongates the telomeric TG(1-3) repeat; and Rad52 is required for both types (Le et al., 1999). Recent data has shown that the APB is not required for ALT in some human cell lines, the telomere length distributions in these cells resembles those of normal ALT cells, while the contents of their telomeres contain a large proportion of non-telomeric

sequences (Fasching et al., 2005), similar to the telomeres of *S. cerevisiae* type 1 survivor cells.

An unusual telomere maintaining mechanism was found in *Drosophila melanogaster* (Biessmann et al., 1990) and some other Dipteran drosophilids (Casacuberta and Pardue, 2003). This involves attachment of non-long-terminal repeat retrotransposons, *HeT-A* and *TART*, to chromosome ends. This transposition-based mechanism results in very heterogeneous and non-specific DNA sequences in the telomeres. Unlike the end-capping protein complex previously discussed, the capping complex in *D. melanogaster* appears to bind to any stable chromosome ends, in a sequence-independent manner (Fanti et al., 1998), suggesting a higher tolerance to telomere-deletion events. A similar mechanism was also suggested in telomerase and RAD52-defective PAL survivors in budding yeast (Maringele and Lydall, 2004).

#### **1.1.5 Regulation of telomerase**

In addition to recruitment by shelterin and CST complex, and factors affecting processivities reviewed in previous sections, telomerase is also regulated by various other mechanisms (reviewed in (Liu et al., 2004b, Flores et al., 2006)). The fact that the transcription of TERT, relative to TERC, varies and correlates to a larger extent with the telomerase activity between mortal and immortal cell lines suggests its prominent and rate-limiting role in telomerase regulation in human (Meyerson et al., 1997). In contrast, expression of TERC in mouse tissues correlates strongly with telomerase activity (Blasco et al., 1995), suggesting different roles of TERT and TERC in telomerase regulation in different organisms. Various binding sites of transcriptional regulators have been identified in the promoter

region of human TERT (hTERT). c-Myc, which is involved in cell proliferation and differentiation (Henriksson and Lüscher, 1996), was found to directly activate hTERT through an E-box site in the promoter (Greenberg et al., 1999, Wu et al., 1999); while its antagonist, Mad, was found to repress hTERT (Günes et al., 2000, Oh et al., 2000). The ratio of c-Myc and Mad in normal somatic cells and immortal cells correlates with their telomerase activities (Oh et al., 2000). Similarly, upstream stimulatory factors (USF) 1 and 2 utilise two E-boxes in hTERT promoter as heterodimers, their activator effect can be enhanced by cofactor p300 and reduced by inhibition of p38 MAP kinase in immortalised cell lines but not in mortal cells (Goueli and Janknecht, 2003). The signal transducer and activator of transcription 3 (STAT3) was also shown to bind directly and activates hTERT promoter, and hTERT expression is required for survival of STAT3-dependent tumour cells (Konnikova et al., 2005). Tissue-specific regulation of hTERT was demonstrated by an estrogen response element in the promoter, which directly associates with the estrogen receptor  $\alpha$ ; together with the activation of c-Myc, estrogen confers specific telomerase up-regulation in mammary and ovarian epithelial cells (Kyo et al., 1999, Misiti et al., 2000). A majority of hTERT repressors currently found belong to tumour suppressors and oncogenic pathways, such as RAK/BRIT1 (Lin and Elledge, 2003) and p53 (through interaction with transcription factor Sp1, which binds to the five GC-boxes in the promoter (Kanaya et al., 2000)). In addition, the human T-cell leukaemia virus type 1 oncoprotein Tax was found to suppress hTERT expression by competing with c-Myc for DNA binding specifically in pre-leukaemic cells (Gabet et al., 2003). The transforming growth factor beta (TGF- $\beta$ ) can also repress hTERT expression, mediated by Smad-interacting protein 1 (SIP1), while Menin directly represses hTERT (Lin and Elledge, 2003). The transcription factor E2F-1,2

and 3, but not E2F-4 and 5, was found to reduce hTERT promoter activity in tumour cells by inhibition of Sp1-mediated activation, in contrast, all E2F factors activate hTERT in normal somatic fibroblast lines through a non-canonical E2F site (Crowe et al., 2001, Won et al., 2002). Additionally, hTERT promoter contains the transcription factor activator protein (AP-1) binding site, which mainly associates with c-Jun, JunD, and c-Fos to suppress hTERT expression (Takakura et al., 2005). Several binding motifs for myeloid-specific zinc finger protein 2 (MZF-2) were also identified in the promoter, and overexpression of MZF-2 in cancer cell lines leads to reduction of hTERT expression and telomerase activity (Fujimoto et al., 2000).

In addition to the promoter-binding factors, the hTERT promoter contains clusters of CpG dinucleotides which are subject to DNA methylation, which can cause transcriptional silencing (Horikawa et al., 1999). However the methylation states between different cells are not consistent with their telomerase activities (reviewed in (Liu et al., 2004b)). While hypermethylation of the hTERT promoter was found to inhibit hTERT expression in normal oral fibroblasts and senescent normal oral keratinocytes (Shin et al., 2003), some undifferentiated and untransformed mortal telomerase-negative cells were found to possess hypomethylated hTERT promoters (Dessain et al., 2000, Lopatina et al., 2003), and some transformed or neoplastic cells have their endogenous hTERT reactivated despite their hypermethylated promoters (Guilleret et al., 2002). These results indicates that the binding of transcriptional regulators to the hTERT promoter is only partly affected by DNA methylation, and this epigenetic influence seems cell type specific.

Alternative splicing of TERT mRNA can affect telomerase activity as well (more detail in section 5.1.1). Although the splicing isoforms include both exon-exclusion and intron-insertion variants, studies have only been done on transcripts with excluded exons. While only the constitutively spliced full length transcript gives rise to catalytic activity (Yi et al., 2000, Ulaner et al., 2000), the isoform hTert $\alpha$ , which contains an in-frame truncation of Motif A in the RT domain, acts as a dominant negative inhibitor of endogenous telomerase activity when overexpressed in immortalised and cancer cell lines (Colgin et al., 2000, Yi et al., 2000). Splicing isoforms were also recently identified in a chicken MDV lymphoma-derived MSB-1 T-cell line (Amor et al., 2010). Isoforms with in-frame exon exclusions were shown to be inactive, and account for the majority of chTERT transcript in normal T-cells, which possess low telomerase activity. Isoforms with premature STOP codons were hypothesised to be subjects of nonsense mediated decay (NMD), this was confirmed in the most abundant variant of this type by RNAi against the NMD pathway. During MDV induced lymphomagenesis, up-regulation of telomerase activity coincides with increased proportion of the constitutively spliced transcript and a shift of inactive transcripts from in-frame variants to NMD-targeted variants (Amor et al., 2010).

Post-translational modifications were also shown to affect telomerase activity. Both hTERT and TP1 proteins are subjects of phosphorylation by Protein Kinase C $\alpha$  (PKC $\alpha$ ) and dephosphorylation by Protein Phosphatase 2A (PP2A) in breast cancer cells (Li et al., 1997, Li et al., 1998). Treatment of breast cancer cell extracts with PP2A causes an inhibition of the cellular telomerase activity, which is reversible with PKC $\alpha$  treatment. Similarly, Akt Protein Kinase was found to phosphorylate and enhance hTERT in a



melanoma cell line (Kang et al., 1999). In addition to tumour cell lines, regulation of hTERT by phosphorylation was also observed in normal human T lymphocytes. Despite the significant difference in telomerase activity between thymocytes and blood peripheral T cells, the transcription levels of hTERT in these cells are equivalent, and up-regulation of telomerase activity during CD4<sup>+</sup> T cell activation does not involve net increase in hTERT protein. Instead, hTERT protein is phosphorylated and recruited into the nucleus upon T cell activation (Liu et al., 2001).

## **1.2 Telomeres and stem cells in planarians**

### **1.2.1 Stem cells of potentially immortal worms**

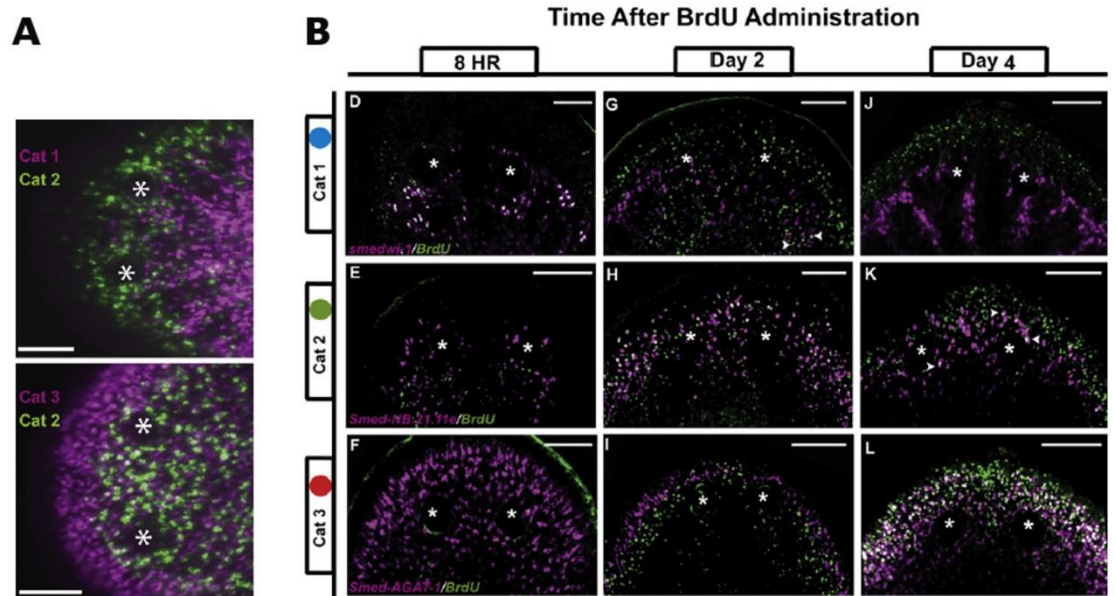
Planarians are free-living flatworms that belong to the phylum Platyhelminthes. Classical experiments have discovered that these organisms have the ability to regenerate complete animals from almost any fragment of the body, given that the fragment contains enough adult somatic stem cells ((Randolph, 1897, Morgan, 1898) reviewed in (Newmark and Alvarado, 2002)). It was found in *Dugesia lugubris* that a fragment as small as 1/279 of the worm is capable of full regeneration. Additionally, fragments from the pharynx and the region anterior to the eyes were found to be post-mitotic and unable to regenerate (Morgan, 1898).

The adult somatic stem cells in planarians, termed the neoblasts (Randolph, 1892), are pluripotent proliferative cells which give rise to the regeneration capacity and long term viability of the species (Baguñà, 1976a, Baguñà, 1976b, Baguñà et al., 1989). During regeneration, neoblasts divide and differentiate into missing tissue types, while the

existing tissues are modified according to the new body plan (Morgan, 1901, Baguñà, 1976b). During the homeostatic cell turnover of intact worms, neoblasts produce progeny which can further differentiate to replace aged cells (Baguñà, 1976b, Pellettieri and Alvarado, 2007, Eisenhoffer et al., 2008). Neoblasts are described as small (5-10  $\mu\text{m}$  in diameter) cells with large nuclei and very little cytoplasm ((Hayashi et al., 2006), reviewed in (Reddien and Alvarado, 2004)), they are scattered throughout the parenchyma, and are capable of migrating to the wound site and form a regeneration surface termed the blastema (Baguñà, 1976b). X-irradiation has been applied as a method to remove proliferative cells in planarians, which results in the inability to form and maintain blastema, loss of regeneration capacity, as well as disruptions of homeostatic functions, which ultimately leads to the death of the organism (Bardeen and Baetjer, 1904). In addition, transplantation of single clonogenic neoblasts into irradiated planarians restores their regeneration capacity and long-term viability, providing evidence for the pluripotency of this cell type (Wagner et al., 2011).

Recent studies of irradiation sensitive cells in planarian, including expression pattern of cell markers (such as *Smedwi-1/2* (Reddien et al., 2005), *Smedwi-3* (Palakodeti et al., 2008) and *Smed-Cbx-1* (Eisenhoffer et al., 2008)) and fluorescence activated cell sorting (FACS) of whole body cells (Hayashi et al., 2006), have revealed various sub-populations of neoblast. Within the irradiation sensitive cells, the X1 population sorted by FACS are proliferative ( $>2N$  DNA content,  $\sim 20\%$  in S phase and  $\sim 75\%$  in G2/M) with a size of approximately  $10\mu\text{m}$  in diameter, while X2 cells are mainly non-cycling ( $\sim 75\%$  in G0/G1) and are  $6\mu\text{m}$  in diameter (Hayashi et al., 2006, Eisenhoffer et al., 2008). Cells in planarian were categorised by

their rate of disappearance after irradiation (Eisenhoffer et al., 2008). The Category 1 cells are eradicated within 24 hours after irradiation, while Category 2 takes two days and Category 3 takes three to four days. Cell lineage tracing using a pulse treatment of 5-bromo-2-deoxyuridine (BrdU), a traceable analogue of thymidine which can be incorporated into the newly replicated genome during S-phase, has demonstrated that the different categories of cells represent the progress of neoblast differentiation towards post-mitotic progenies ((Eisenhoffer et al., 2008), see Figure 1.5). When combined with FACS, the X1 population contains a high proportion of cells expressing Category 1 markers (30% - 80%, varied between each marker), while X2 contains a mixture of category cells (23% - 44% Category 1, 8.5% Category2, and 12% Category 3). It is uncertain whether cells expressing different Category 1 markers with various expression patterns differ in their tissue specificities.



**Figure 1.5 – Anterior distribution and lineage tracing of category cells.** **(A)** Fluorescent in-situ hybridisation with category markers (Cat1: *Smed-wi1*; Cat2: *NB.21.11e*; Cat3: *Smed-AGAT1*) showing the spatial distribution of stem cells and descendants at the anterior end of *S. mediterranea*. **(B)** Localisation of category markers after a single pulse of BrdU treatment, showing the progressive anterior migration of BrdU positive cells and the co-localisation of BrdU and category markers in a time-specific manner. White dots refer to cells with overlapping marker signals. \*: Photoreceptors. Scale bar: 100µm. (Adapted from Eisenhoffer et al., 2008)

### 1.2.2 A simple planarian model for complex questions

The model planarian species used in this PhD project, *Schmidtea mediterranea*, has the ability to derive clonal lines from single worms due to its regeneration and homeostatic capacity, given that appropriate nutrients are provided. The species is divided into asexual and sexual strains, which differ genomically by a translocation between Chromosome I and III in the asexual strain (Baguña et al., 1999). The sexual strain is

hermaphroditic, its germ cells are specified at no later than stage 8 during embryogenesis (Handberg-Thorsager and Salo, 2007), and develop into gamete-producing testes and ovaries in mature adults. However the adult somatic stem cells seem also capable of differentiating into germ cells, since *nanos*-positive germ cells are reconstituted in regenerating worms recovered from amputated body fragments deprived of *nanos*-expressing cells (Wang et al., 2007b). The asexual strain lacks reproductive organs, and reproduces by fission, in which a segment most posterior of the tail is pulled off from the body, followed by regeneration of the two body parts into two individual worms. This reproductive cycle is constantly repeated throughout the evolution history of the asexual strain, suggesting that the soma has become an effective inheritable unit which can propagate without need to passage through the germline. Therefore powerful cellular maintenance mechanisms must exist in planarians, with which telomeres must not erode, oxidative stresses must be removed, and DNA damage must be sufficiently repaired in order to prevent stem cell depletion after finite population doublings.

It is not certain whether stem cells in the sexual strain also possess this immortal property, some early research has suggested limited life span in closely related sexual planarian species, such as *Phagocata velata* (Child, 1914) and *Dugesia lugubris* ((Haranghy and Balázs, 1964) and citations within). Recent work in a sexual marine flatworm *Macrostomum lignano* has provided an accurate demography which shows the limited lifespan of this species (Mouton et al., 2009). It has been suggested that in multicellular organisms which sequester germ lines, the function of the soma is to increase the possibility of successful inheritance of germline to the next generation, and is susceptible to senescence after reproduction is

complete (Weismann, 1891). A common theory for ageing in multicellular organisms is the pleiotropic effect of genes which are naturally selected to maximise the fitness of soma at the sexually reproductive stage of life, while being deleterious at later stages since fitness of the soma after reproduction may not be the subject of natural selection (Williams, 1957). Alternatively, ageing may be selected for in a population with limited resources, in order to prevent inter-generational competition and maximise the survival of offspring (Weismann, 1891). Since offspring contain diverse genetic combinations arisen from meiotic recombination and mating, and the population relies on these variations to defend against constantly-evolving environmental challenges such as parasites and pathogens (Red Queen hypothesis, (Hamilton, 1980)), ageing may act as a mechanism to prevent older individuals from dominating the gene pool (Goldsmith, 2008). The study of ageing in planarians has not been pursued with molecular tools. *S. mediterranea* possess naturally occurring strains with different reproduction strategies, which can be used for testing the link between sexual reproduction and somatic senescence.

The haploid genome of *S. mediterranea* consists of approximately 865Mb of A-T rich DNA sequence, distributed in 4 chromosomes ((Baguña et al., 1999, Prats, 1991), reviewed in (Newmark and Alvarado, 2002)). Compared to other planarian species that have more complex karyotypes (such as 15 chromosomes in *S. polychroa*) and ploidies (such as *Dugesia lugubris* which has triploid somatic cells, hexaploid female germ cells, and diploid male germ cells (Benazzi, 1957)), *S. mediterranea* has a stable diploid genome which can be easier to observe and study. The genome of the sexual *S. mediterranea* has been sequenced with moderate coverage (11.6 fold) (Washington University Genome Center and (Blythe et al.,

2010)), and is ideal for both bioinformatic and experimental studies. However the sequence assembly is challenged by the AT-rich (greater than 65%) nature and the repetitiveness (more than 40%) of the genome (Blythe et al., 2010).

### 1.2.3 About this project

The planarian telomeric repeat was originally identified in *Polycelis tenuis*, which contains the same repeat unit as the vertebrates, TTAGGG (Joffe et al., 1996). Preliminary bioinformatic analysis has identified several sequencing reads that may belong to the telomere and sub-telomeric regions (see section 3.1.1). In this PhD project, the (TTAGGG)<sub>n</sub> repeat in *S. mediterranea* was confirmed to be telomeric using Bal-31 exonuclease digestion followed by TRF analysis. Given the cell cycle dependent nature of the canonical telomerase mechanism, it is likely that a high telomere maintenance activity can be observed in worms during regeneration, when peaks of cell proliferation have been previously documented. To test this theory, telomere length dynamics were compared between homeostatic and regenerating samples from both sexual and asexual strains using a restriction digest-based and a qPCR-based technique. In order to confirm the activity of canonical telomerase enzyme, the telomere repeat amplification protocol (TRAP) assay was applied using total protein extracts from regenerating asexual samples. The same technique was used to compare the telomerase activity between homeostatic and regenerating samples of the two strains (see Chapter 3). The gene encoding the telomerase catalytic subunit, *Smed\_Tert*, was identified and characterised in *S. mediterranea*. The spatial pattern of the gene expression was visualised by *in-situ* hybridisation. The function of *Smed\_Tert* was studied using RNA interference (see Chapter 4). Four isoforms of *Smed\_Tert* were

discovered by cloning of reverse transcription (RT)-PCR products, with one being the full-length transcript and the other three containing specific exon exclusions. Ratios of these isoforms in the transcriptome were measured with qRT-PCR and compared between the strains in intact and regenerating samples (see Chapter 5). With an aim to study the telomere dysfunctional phenotype, a shelterin component homolog, *Smed\_Pot1*, was also identified and preliminarily characterised (see Chapter 6). Taken all together, *S. mediterranea* is a suitable model for studying how multicellular organisms can evolve to possess an immortal soma. It also provides an *in vivo* environment for studying telomere dynamics and telomere-related protein function in a native stem cell niche, and in a regenerative context.



## CHAPTER 2: MATERIALS AND METHODS

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### 2.1 Materials

#### 2.1.1 Planarian culture

Both the asexual and the hermaphroditic sexual strains of *Schmidtea mediterranea* were cultured by Mr. Jamie Jowett at 20°C using tap water filtered through activated charcoal and buffered with 0.5mM sodium hydrogen carbonate (Sigma). The worms were fed organic veal liver once per week, and were starved for at least 7 days prior to analysis or amputation. For the duration of experiments the worms were treated with 10mg/ml gentamicin (Fluka) to prevent bacterial infections.

#### 2.1.2 Oligonucleotides

All oligonucleotides were synthesised and desalted by Sigma-Aldrich, with details as follows (PCR linker sequences are shown in lower case):

| Description   | Primer name | Sequence (5' → 3')                      |
|---|-------------|---|
| Amplifies the region for <i>Smed_Tert</i> dsRNA synthesis and in-situ hybridisation | TERT_F2     | ggccgcggCTTCAATATGTTGATGATGTT<br>TTATTC |
|   | TERT_R      | gccccggccAATGGAGAATCCATTTTCATT<br>TGACC |
| Universal secondary PCR primer for adding T7 binding sites                          | Uni_T7_5'   | GAGAATTCTAATACGACTCACTATAGg<br>gccgcgg  |
|   | Uni_T7_3'   | AGGGATCCTAATACGACTCACTATAGg             |

|   |                       |   |
|---|-----------------------|---|
|   |                       | ccccggc   |
| Generation of pure staggered (TTAGGG) <sub>n</sub> dsDNA                | (TTAGGG) <sub>3</sub> | TTAGGGTTAGGGTTAGGG  |
|   | (CTAACC) <sub>3</sub> | CTAACCCTAACCCTAACC  |
| Primer for first strand cDNA synthesis                                  | OligodT18             | GCGAGCACAGAATTAATACGACTCACT<br>ATAGGTTTTTTTTTTTTTTTTTTTVN |
| Amplifies the ORF of the full length transcript                         | TERT_F7               | ggccgcggATGGTTTTATGAAATTAGAT<br>CTTGG                     |
|   | TERT_R                | As above  |
| qPCR for the total transcription level of all <i>Smed_Tert</i> isoforms | TERT_qPCR_F3          | TTATCGAGATTTGCAGGATT                                      |
|   | TERT_qPCR_R3          | CACTACAGCAATTGTCATGG                                      |
| qPCR for transcription level of Alt Region I +ve isoforms               | TERT_qPCR_Alt I_F1    | TCTCGGCGATATTTTCTAA                                       |
|   | TERT_qPCR_Alt I_R1    | TCTTCATTGACTTGCATACG                                      |
| qPCR for transcription level of Alt Region I -ve isoforms               | TERT_qPCR_Alt I_F2    | CAAAAACAAGTGTAGTGAAATTAAA                                 |

|  |                        |   |
|--|------------------------|---|
|  | TERT_qPCR_Alt<br>I_R2  | CACCAGTGAAAATTTTGTTGA                       |
| qPCR for transcription<br>level of Alt Region II<br>+ve isoforms   | TERT_qPCR_Alt<br>II_F1 | CTGATTTGATTTCGAAGACTAAAG                    |
|  | TERT_qPCR_Alt<br>II_R1 | GAGGTATTCCGCATATTTGA                        |
| qPCR for transcription<br>level of <i>cystatin</i><br>(internal control)   | Cys_qPCR_F             | AACTCCATGGCTAGAACCGAA                       |
|  | Cys_qPCR_R             | CCGTCGGGTAATCCAAGTACA                       |
| qPCR for telomere<br>repeat copy number in<br>gDNA   | Tel_qPCR_F             | GGTTTTTGAGGGTGAGGGTGAGGGTG<br>AGGGTGAGGGT   |
|  | Tel_qPCR_R             | TCCCGACTATCCCTATCCCTATCCCTA<br>TCCCTATCCCTA |
| qPCR for single copy<br>gene NB.21.11e in<br>gDNA (internal control)<br>and as marker for<br>Category 2 gene<br>expression | NB.21.11e_F            | GTCTCCCGCCAAATCAAGTA                        |
|  | NB.21.11e_R            | TTTCATGCAATCTGCTTTTCG                       |
| qPCR marker for<br>Category 1 gene<br>expression   | Smedwi-2_F             | CTGCAAAAAGATTACCTATGCTCTAACT                |
|  | Smedwi-2_R             | ACGGGATTAGAGCCCTTATGCAC                     |

|   |              |  |
|---|--------------|--|
|   |              |  |
| qPCR marker for<br>Category 3 gene<br>expression                | Agat1_F      | GTTGGTTGAAAAGCGAGAGGTT                 |
|   | Agat1_R      | CGAACATCGGAAGTCCAACAATG                |
| Amplifies the region for<br><i>Smed_Pot1</i> dsRNA<br>synthesis | POT1_F       | ggccgcggAAATACAGCCACGTTGAATC<br>CT     |
|   | POT1_R1      | gccccggccTACGATTTTACTGCTTTAAC<br>AGATG |
| 3' RACE Outer reverse<br>primer (kit)                           | 3'Out_R      | GCGAGCACAGAATTAATACGACT                |
| 3' RACE Outer forward<br>primer ( <i>Smed_Tert</i> )            | TERT_3'Out_F | TTATCGAGATTTGCAGGATT                   |
| 3' RACE Outer forward<br>primer ( <i>Smed_Pot1</i> )            | POT1_3'Out_F | TACGATTTTACTGCTTTAACAGATG              |
| 3' RACE Inner reverse<br>primer (kit)                           | 3'Inn_R      | CGCGGATCCGAATTAATACGACTCACT<br>ATAGG   |
| 3' RACE Inner forward<br>primer ( <i>Smed_Tert</i> )            | TERT_3'Inn_F | CTGTCTAGAGCCCCATGAAT                   |
| 3' RACE Inner forward<br>primer ( <i>Smed_Pot1</i> )            | POT1_3'Inn_F | CCTGAAAATCTCCCTAGGAT                   |

|   |              |   |
|---|--------------|---|
| 5' RACE RNA Adaptor                               |              | GCUGAUGGCGAUGAAUGAACACUGC<br>GUUUGCUGGCUUUGAUGAAA |
| 5' RACE Outer forward primer (kit)                | 5'Out_F      | GCTGATGGCGATGAATGAACACTG                          |
| 5' RACE Outer reverse primer ( <i>Smed_Tert</i> ) | TERT_5'Out_R | TTTTTACAAGACATCGAGCA                              |
| 5' RACE Outer reverse primer ( <i>Smed_Pot1</i> ) | POT1_5'Out_R | AAATACAGCCACGTTGAATC                              |
| 5' RACE Inner forward primer (kit)                | 5'Inn_F      | CGCGGATCCGAACACTGCGTTTGCTG<br>GCTTTGATG           |
| 5' RACE Inner reverse primer ( <i>Smed_Tert</i> ) | TERT_5'Inn_R | CGCCTGAATAAGAATTTGAC                              |
| 5' RACE Inner reverse primer ( <i>Smed_Pot1</i> ) | POT1_5'Inn_R | AAATACAGCCACGTTGAATCCT                            |
| M13 primers                                       | M13_F        | GTAAACGACGGCCAGT                                  |
|   | M13_R        | GCGGATAACAATTTACACAGG                             |
| <i>Mariner1</i> , target for TAR cloning          | Mar1_F       | tacgcgtcgacTAGTTTCCTGATTCCATCG<br>GCG             |
|   | Mar1_R       | tacgcgtcgacggatccTGGATATTCTGCC                    |

|  |  |        |
|--|--|--------|
|  |  | GCAGGG |
|--|--|--------|

### 2.1.3 Genomic contigs and trace sequencing reads

All genomic contigs used in bioinformatic analysis were obtained from the sexual *S.mediterranea* genome assembly (version 3.1, Washington University Genome Center, [http://genome.wustl.edu/genomes/view/schmidtea\\_mediterranea/](http://genome.wustl.edu/genomes/view/schmidtea_mediterranea/)), while the trace reads were acquired from the NCBI trace archive (<http://www.ncbi.nlm.nih.gov/Traces/home/>).

### 2.1.4 TERT protein sequence in multiple species

Sequences for multi-species TERT alignment were obtained from the NCBI RefSeq database (<http://www.ncbi.nlm.nih.gov/protein>) with the following details:

| Species                         | Protein name | Accession   |
|---------------------------------|--------------|-------------|
| <i>Caenorhabditis elegans</i>   | Trt-1        | NP_492373.1 |
| <i>Homo sapiens</i>             | TERT         | O14746.1    |
| <i>Fugu rubripes</i>            | TERT         | AAX59693.1  |
| <i>Tetrahymena thermophila</i>  | TERT         | O77448.1    |
| <i>Saccharomyces cerevisiae</i> | Est2p        | Q06163.1    |

### 2.1.5 POT1 protein sequence in multiple species

The following sequences of POT1 were obtained from the NCBI RefSeq database:

| Species                          | Protein name   | Accession    |
|----------------------------------|----------------|--------------|
| <i>Schizosaccharomyces pombe</i> | POT1           | CAB16192     |
| <i>Arabidopsis thaliana</i>      | AtPot1         | AAX78213     |
| <i>Xenopus laevis</i>            | POT1           | NP_001084422 |
| <i>Gallus gallus</i>             | POT1           | NP_996875    |
| <i>Mus musculus</i>              | POT1a          | NP_598692    |
| <i>Homo sapiens</i>              | POT1 isoform 1 | NP_056265    |

### 2.1.6 Buffers and solutions

#### 1x TE pH 8.0:

10 mM Tris-HCl, 1 mM EDTA

#### 10x TBE pH 8.3:

890mM Tris, 890mM boric acid, 20mM EDTA

#### 2.1.6.1 For Southern hybridisation

##### Church-Gilbert Buffer:

0.5M Sodium phosphate buffer pH7.2, 1mM EDTA, 7% SDS

##### Denaturing solution:

1M NaCl, 0.5M NaOH

##### 20x SSC pH7.0:

3M NaCl, 0.3M sodium citrate

### *2.1.6.2 For in-situ hybridisations and immunohistochemistry*

#### **10x DIG rNTPs:**

3.57mM DIG-UTP, 6.43mM UTP, 10mM ATP, 10mM GTP, 10mM CTP

#### **10x PBS pH 7.2-7.4:**

1.369M NaCl, 26.827mM KCl, 14.696mM KH<sub>2</sub>PO<sub>4</sub>, 80.997mM anhydrous Na<sub>2</sub>HPO<sub>4</sub>

#### **5/8 Holtfreter's solution:**

0.374M NaCl, 0.416M KCl, 0.567mM CaCl<sub>2</sub>, 1.488mM NaHCO<sub>3</sub>

#### **Carnoy solution [made fresh]:**

60% ethanol, 30% chloroform, 10% acetic acid

#### **Prehybridisation buffer:**

50% formamide, 5x SSC, 0.1mg/ml yeast total RNA (Ambion), 0.1mg/ml heparin (Roche), 0.1% Tween20; 10mM DTT

#### **Hybridisation buffer [made fresh]:**

10% dextran sulphate in prehybridisation buffer

#### **Proteinase K digestion buffer:**

50mM Tris pH 8.0, 10mM EDTA, 0.5% SDS

#### **PostHyb1 solution [made fresh]:**

50% formamide, 5x SSC

#### **PostHyb2 solution [made fresh]:**

75% PostHyb1 solution, 25% PostHyb5 solution

#### **PostHyb3 solution [made fresh]:**

50% PostHyb1 solution, 50% PostHyb5 solution

#### **PostHyb4 solution [made fresh]:**

25% PostHyb1 solution, 75% PostHyb5 solution

#### **PostHyb5 solution [made fresh]:**

2x SSC, 0.1% TritonX-100

#### **PostHyb6 solution [made fresh]:**



0.2x SSC, 0.1% TritonX-100

**Buffer I pH7.5 [made fresh]:**

0.1% TritonX-100, 99.914mM maleic acid, 0.167M NaCl, pH adjusted with NaOH

**Buffer II [made fresh]:**

1% Blocking Reagent (Boehringer Mannheim) in Buffer I

**Resuspension buffer:**

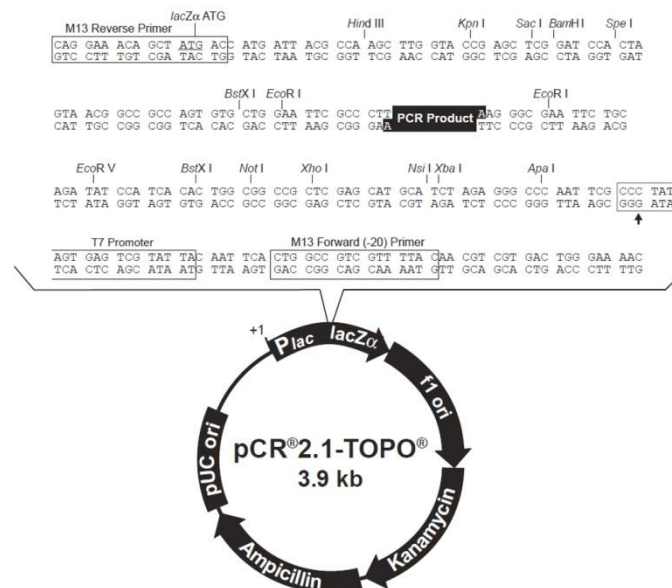
5ml Formamide + 5ml 1x TE pH7.5 + 10µl 0.1% Tween20

**TMN solution [made fresh]:**

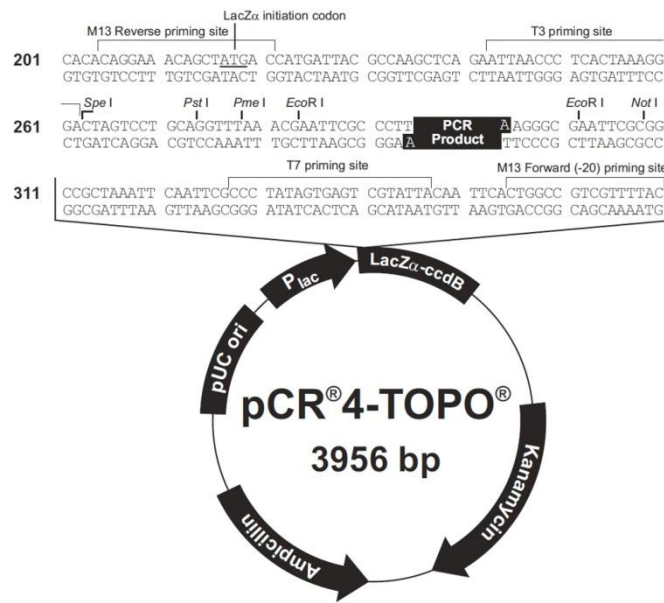
0.1M Tris-HCl pH9.5, 0.1M NaCl, 0.05M MgCl<sub>2</sub>, 1% Tween20, 10% polyvinyl alcohol (98-99%, hydrolysed)

## 2.1.7 Vectors used in TA cloning

**pCR2.1-TOPO (Invitrogen)**



## pCR4-TOPO (Invitrogen)



## 2.2 Methods

### 2.2.1 RNA interference - *Smed\_Tert*

A 601bp section located across the Motif C, D, and E of the *Smed\_Tert* transcript (PCR product from primer TERT\_F2 and TERT\_R) was amplified from first strand cDNA synthesised from total RNA extracted from asexual *S. mediterranea* (see section 2.2.10) using a gene specific primer set with 5' linkers (forward primer linker: 5'-ggccgcgg-3'; reverse primer linker: 5'-gccccgcc-3') which act as binding sites for a ubiquitous secondary PCR primer set, which attaches a T7 RNA polymerase binding site at the 5' end of both strands (see section 2.1.2 for primer sequences). The secondary PCR product was used as a template for in vitro transcription with T7 RNA polymerase (Roche), the conditions of PCR and transcription reactions are listed as follows.

**Primary PCR with gene-specific primers:**

| Total reaction volume: 50µl  |                     | Cycling conditions/procedures                                   |             |          |
|------------------------------|---------------------|---|-------------|----------|
| Reagent                      | Final concentration | Step  | Temperature | Duration |
| 10x NH4 Taq Buffer (Bioline) | 1x                  | 1   | 94°C        | 1 min    |
| 50mM MgCl <sub>2</sub>       | 1.5mM               | 2   | 94°C        | 30 sec   |
| 2mM dNTP                     | 0.2mM               | 3   | 54°C        | 30 sec   |
| Primer: TERT_F2 (10µM)       | 300nM               | 4   | 72°C        | 1 min    |
| Primer: TERT_R (10µM)        | 300nM               | Repeat  | Step 2-4    | 34 Times |
| cDNA                         | 1ng/µl              | 5   | 72°C        | 2 min    |
| Taq polymerase (Bioline)     | 0.06U/µl            | PCR product was gel-extracted with a Qiagen gel-extraction kit. |             |          |
| Deionised water              | Fill to 50µl        |   |             |          |

### Secondary PCR with universal T7 primers:

| Total reaction volume: 50µl    |                     | Cycling conditions  |             |          |
|--------------------------------|---------------------|---|-------------|----------|
| Reagent                        | Final concentration | Step  | Temperature | Duration |
| 10x NH4 Taq Buffer (Bioline)   | 1x                  | 1   | 94°C        | 1 min    |
| 50mM MgCl <sub>2</sub>         | 1.5mM               | 2   | 94°C        | 30 sec   |
| 2mM dNTP                       | 0.2mM               | 3   | 55°C        | 30 sec   |
| Primer:<br>Uni_T7_5'<br>(10µM) | 300nM               | 4   | 72°C        | 1 min    |
| Primer:<br>Uni_T7_3'<br>(10µM) | 300nM               | Repeat  | Step 2-4    | 34 Times |
| Purified primary PCR product   | 0.02ng/µl           | 5   | 72°C        | 2 min    |
| Taq polymerase (Bioline)       | 0.06U/µl            | PCR product was checked on gel and purified with a Qiagen PCR purification kit. |             |          |
| Deionised water                | Fill to 50µl        |   |             |          |

**In vitro transcription:**

| Total reaction volume: 40µl           |                     | Procedure   |
|---------------------------------------|---------------------|---|
| Reagent                               | Final concentration | Incubated at 37°C for 8.5 hours.  |
| 10x transcription buffer (Roche)      | 1x                  | Added 2 units of TURBO DNase (Ambion).  |
| 20mM rNTP                             | 3mM                 | Incubated at 37°C for 30 minutes.   |
| Secondary PCR product                 | 20ng/µl             | Added 2.5µg of glycogen, precipitated with 510µl of EtOH at -20°C overnight.      |
| RNAseOUT RNAse inhibitor (Invitrogen) | 1U/µl               | Centrifuged at 12,000 x g for 30 minutes, supernatant removed.                    |
| T7 RNA polymerase (Roche)             | 2U/µl               | Pellet washed with 1ml of 70% EtOH, then resuspended in 25µl of RNAse-free water. |
| RNAse-free water                      | fill to 40µl        |   |

1:80, 1:40, 1:20 and 1:10 dilutions of the final product were run on a 1% agarose gel in 1 x TBE, and quantified using band intensity readings with the Quantity One software (BioRad); Q-step 4 DNA ladder (1425 ng/lane,

YOUBIO) was used as a quantitative standard. The double-stranded RNA was then diluted to 2 µg/µl, and 96.6nl (3 x 32.2nl) was injected into the gut branch of each planarian from the ventral side anterior to the pharynx using the Nanoject II (Drummond Scientific) apparatus. The 3.5" glass capillaries (Drummond Scientific) were pulled using a Flaming/Brown Micropipette Puller (Patterson Scientific), with the following settings: heat 295, time 150, pull 200, and velocity 120. The experiment consisted of injections on three consecutive days of the first week, followed by maintenance injections at a frequency of twice a week. The negative control samples were injected with the same amount of dsRed double-stranded RNA, of which no homologous gene is present in *S. mediterranea*. For telomere length analysis and TRAP assay, all samples were amputated, followed by extractions at 72 hours post-cut.

### 2.2.2 RNA interference – *Smed\_Pot1*

Double strand RNA was synthesised *in vitro*, using the same procedure as section 2.2.1, from a 309bp RT-PCR product which locates at the OB1 fold encoding region of the transcript. The conditions for the primary PCR are listed as follows:

| Total reaction volume: 50µl  |                     | Cycling conditions |             |          |
|------------------------------|---------------------|--------------------|-------------|----------|
| Reagent                      | Final concentration | Step               | Temperature | Duration |
| 10x NH4 Taq Buffer (Bioline) | 1x                  | 1                  | 94°C        | 1 min    |
| 50mM MgCl <sub>2</sub>       | 1.5mM               | 2                  | 94°C        | 30 sec   |

|                             |              |  |        |          |          |
|-----------------------------|--------------|--|--------|----------|----------|
| 2mM dNTP                    | 0.2mM        |  | 3      | 55°C     | 30 sec   |
| Primer:<br>POT1_F (10µM)    | 300nM        |  | 4      | 72°C     | 1 min    |
| Primer:<br>POT1_R1 (10µM)   | 300nM        |  | Repeat | Step 2-4 | 34 Times |
| cDNA                        | 1ng/µl       |  | 5      | 72°C     | 2 min    |
| Taq polymerase<br>(Bioline) | 0.06U/µl     |  |        |          |          |
| Deionised water             | Fill to 50µl |  |        |          |          |

The RNA was diluted to 2 µg/µl and 96.6nl was injected into the gut of each planarian as described above. The injection was done on three consecutive days per week, for a total of two weeks. The worms were amputated a day after the final injection. Homeostatic phenotypic observation was done with worms injected with the same regime and left without amputation. Control samples were injected with the same amount of dsRed double-stranded RNA.

### **2.2.3 Genomic DNA extraction**

Worms were frozen at -20°C overnight in 300µl of lysis buffer (110mM NaCl, 110mM Tris-HCl pH8.5, 55mM EDTA pH8.0, and 1.14% SDS), followed by thawing and homogenising with a sterilised pestle on the second day. The pestle was washed with 200µl of lysis buffer, which was added into the homogenised sample. The 500µl sample was then digested with 0.1 mg/ml Proteinase K and 0.1 mg/ml RNaseA at 37°C for 30 minutes, then 65°C for 30 minutes. The genomic DNA was purified with two basic Phenol (pH 8.0) extractions, followed by removal of residual phenol with a chloroform extraction, all centrifugation steps were done at 4°C. The sample was added with 4µg of glycogen, and then precipitated with 1ml of EtOH at -20°C overnight. The sample was centrifuged at 12,000 x g for 45 minutes at 4°C, after which the supernatant was removed, and the pellet was washed twice in 1ml of 70% EtOH. The final product was resuspended in 1 x TE pH8.0, and quantified on a 1% agarose gel in 1 x TBE, as described in section 2.2.1. The DNA was stored at 4°C.

### **2.2.4 Effect of regeneration on telomere length**

For each sample, an intact worm was cut into head, middle, and tail pieces. gDNA from the tail piece was extracted immediately after the cut while the remaining pieces were allowed to regenerate. The regenerants were further cut 5 days and 10 days after the first amputation. gDNA was extracted after 3 rounds of regeneration.



### 2.2.5 TRF-Southern analysis

0.65µg of genomic DNA was digested to completion by AluI, HaeIII, and MboI using the following procedure:

| Total reaction volume: 150µl |                     | Procedures  |
|------------------------------|---------------------|---|
| Reagent                      | Final concentration | Incubated at 37°C for 16 hours.                                 |
| 10x NEB Buffer 2             | 1x                  | Added with 9µl of 3M sodium acetate pH 5.2 and 4µg of glycogen. |
| MboI                         | 20U total           | Precipitated with 500µl of EtOH at -20°C overnight.             |
| AluI                         | 20U total           | Centrifuged at 12,000 x g for 30 minutes, supernatant removed.  |
| HaeIII                       | 20U total           | Pellet washed with 70% EtOH.                                    |
| gDNA                         | 650ng total         | Resuspended in 22µl of 1 x TE pH 8.0.                           |
| Deionised water              | Fill to 150µl       |   |

FIGE pulsefield electrophoresis was performed with the separation optimised for a range between 2kb and 35kb (see section 2.2.7 for details about FIGE settings). The DNA was denatured in the denaturing solution, transferred to a quaternary amine derivatised nylon membrane (Zeta-Probe GT, Bio-Rad) in the denaturing solution using a VacuGene™ XL Vacuum Blotting System (Amersham), and probed with <sup>32</sup>p labelled telomeric tandem repeat sequence produced by random primer labelling of a PCR product from a primer set which yields staggered pure (TTAGGG)<sub>n</sub>

sequence (Forward primer: (TTAGGG)<sub>3</sub>; Reverse primer: (CTAACC)<sub>3</sub>, gel-purified between 300bp – 2kb), the PCR was performed with the following conditions:

| Total reaction volume: 50µl                |                     | Cycling conditions |             |          |
|--|---------------------|--------------------|-------------|----------|
| Reagent                                    | Final concentration | Step               | Temperature | Duration |
| 10x NH4 Taq Buffer (Bioline)               | 1x                  | 1                  | 94°C        | 1 min    |
| 50mM MgCl <sub>2</sub>                     | 1.5mM               | 2                  | 94°C        | 30 sec   |
| 2mM dNTP                                   | 0.2mM               | 3                  | 55°C        | 30 sec   |
| Primer:<br>(TTAGGG) <sub>3</sub><br>(10µM) | 300nM               | 4                  | 72°C        | 1 min    |
| Primer:<br>(CTAACC) <sub>3</sub><br>(10µM) | 300nM               | Repeat             | Step 2-4    | 34 Times |
| Taq polymerase<br>(Bioline)                | 0.06U/µl            | 5                  | 72°C        | 2 min    |
| Deionised water                            | Fill to 50µl        |                    |             |          |

The Labelling reaction was done using the Prime-It® RmT Random Primer Labelling Kit (Stratagene) with the following protocol:

| Reagents   | Quantity        |              |
|--|-----------------|--------------|
|  | (TTAGGG)n probe | Ladder probe |
| (TTAGGG)n PCR product  | 50ng            | 0            |
| λ-HindIII digested DNA   | 0               | 2ng          |
| 5kb size standard<br>(BioRad)                                  | 0               | 2ng          |
| Reaction mix   | 1 vial          | 1 vial       |
| Deionised water  | Fill to 44µl    | Fill to 42µl |
| Denatured at 100°C for 5 minutes                               |                 |              |
| α- <sup>32</sup> p dCTP 3000Ci/mmol<br>10mCi/ml (Perkin-Elmer) | 3µl             | 5µl          |
| Magenta polymerase<br>(Stratagene)                             | 3µl             | 3µl          |
| Incubated at 37°C for 20 minutes                               |                 |              |

|  |     |     |
|--|-----|-----|
| STOP solution  | 2µl | 2µl |
| Labelled probes were purified with Bio-spin 30 column (BioRad). 1µl of the ladder probe was added to the (TTAGGG) <sub>n</sub> probe, the mixture was denatured at 100°C for 5 minutes, and chilled on ice for 5 minutes immediately before use. |     |     |

The membrane was pre-hybridised in Church-Gilbert Buffer at 64°C for one hour, followed by addition of the probe mix to the pre-hyb buffer and hybridised at 65°C overnight. The membrane was washed four times in 3xSSC/0.1%SDS at 64°C. The signal was visualised with a Fujifilm Super RX film and digitalised with an Epson V350 Photo scanner. The image was analysed with the Quantity One software (Invitrogen) and the mean telomere length was defined as  $\Sigma(\text{ODi}) / \Sigma(\text{ODi}/\text{Li})$  as previously described (Harley et al., 1990), where an ODi is the optical density reading of one of the evenly distributed blocks in the lane, and Li is the mean DNA size of the same block.

#### 2.2.6 Bal-31 digestion followed by 6-cutter TRF analysis

800ng of gDNA was treated with 1.5 units of exonuclease Bal-31 in a reaction volume of 50µl for various durations (30, 60, 120, and 240 minutes), followed by inactivation of the enzyme at 65°C for 15 minutes in the presence of 20mM EGTA, and ethanol precipitation subsequently. The samples were resuspended in 1 x NEB Buffer 3 and then digested with a cocktail of 6-cutter restriction enzymes (including EcoRI, MluI, NruI, ScaI and PvuII, 10 units of each enzyme per sample) in a reaction volume of 150µl, for 16 hours. The digested samples were ethanol precipitated and resuspended in 22µl of 1 x TE pH8.0. Separation was done with FIGE,

followed by the TRF southern hybridisation as described in section 2.2.5. For the internal gene control, the digested samples were probed with a <sup>32</sup>p-labelled PCR product of *nanos* gene.

### 2.2.7 Field Inversion Gel Electrophoresis (FIGE)

DNA samples in the TRF analysis were separated with a FIGE Mapper Electrophoresis System (Bio-Rad), using a customised program optimised for separation between 2 – 35 kb with the following settings:

|                 |                   |
|-----------------|-------------------|
| Agarose         | 1.0%              |
| Buffer          | 0.5 TBE           |
| Temperature     | Room temperature  |
| Forward voltage | 180               |
| Reverse voltage | 120               |
| Switch time     | 0.1 – 0.3 seconds |
| Ramp            | 21p               |
| Run time        | 15.8 hours        |

### 2.2.8 Telomere length measurement by qPCR

The primers and cycling parameters were derived from Cawthon (Cawthon, 2002). Reactions were done using Brilliant qPCR Master Mix (Agilent) with a Corbett Rotor-Gene 6000 qPCR machine. gDNA samples were diluted to 10ng/μl and 30ng was used in each qPCR reaction. For telomere copy number, the final primer concentrations were: Tel\_qPCR\_F (270nM); Tel\_qPCR\_R (900nM), and the cycling was done with an initial incubation at 95°C for 10 minutes, proceeded by 35 cycles of 95°C for 15 seconds and 54°C for 2 minutes. For the single copy internal control gene NB.21.11e, 300nM of final concentration was used for both primers, the

cycling was 95°C for 10 minutes, followed by 40 cycles of 95 °C for 15 seconds, 55°C for 20 seconds, and 72°C for 20 seconds. Each sample was analysed in triplicate.

### 2.2.9 Telomere repeat amplification protocol (TRAP) assay

The assay was performed with the TRAPeze® Telomerase Detection Kit (Millipore). For each sample, 3 worms (approximately 8mm long) were snap-frozen with liquid nitrogen in 110µl of the CHAP buffer, with an addition of 200 U/µl of RNasin® Plus RNase Inhibitor (Promega). When thawed, the sample was homogenised on ice with an RNase-free pestle, and was incubated on ice for 30 minutes. The suspension was then centrifuged at 12,000 x g for 20 minutes at 4°C, and the supernatant was collected and quantified with Bradford assay. 0.5µg of the total protein was incubated with the TS Primer in 1x TRAP buffer, in presence of 50µM dNTP, for 45 minutes at 30°C. The product DNA was purified with phenol/chloroform/isoamyl alcohol (25/24/1, pH8.0) followed by precipitation on dry ice with ethanol in presence of 2.5M ammonium acetate, desalt with 70% ethanol, and resuspension in 20µl of deionised water. PCR amplification of the product DNA was performed with the following conditions:

| Total reaction volume: 50µl |                     | Cycling conditions |             |          |
|-----------------------------|---------------------|--------------------|-------------|----------|
| Reagent                     | Final concentration | Step               | Temperature | Duration |
| 10x TRAP Buffer             | 1x                  | 1                  | 94°C        | 1 min    |

|                           |           |  |        |          |          |
|---------------------------|-----------|--|--------|----------|----------|
| 2.5mM dNTP                | 50μM      |  | 2      | 94°C     | 30 sec   |
| 50x TS Primer             | 1x        |  | 3      | 59°C     | 30 sec   |
| 50x Primer Mix            | 1x        |  | 4      | 72°C     | 90 sec   |
| Taq polymerase<br>(Roche) | 0.04 U/μl |  | Repeat | Step 2-4 | 34 Times |
|                           |           |  | 5      | 72°C     | 2 min    |

For each experiment, a positive control reaction was done with the same procedures using a telomerase-positive HeLa cell extract attached with the kit, while a negative control was performed using CHAP buffer only. The PCR products were run in a 10% non-denaturing PAGE gel (in 0.5x TBE), stained with SYBR® Green I (Invitrogen), and visualised with a Fuji FLA2000 phosphorimager.

#### 2.2.10 Total RNA extraction and first strand cDNA synthesis

For each sample, planarians (5 individuals, each with a body length of approximately 5mm, or fewer worms with larger sizes) were snap-frozen in 300μl of TRIzol reagent (Invitrogen) in dry ice and stored at -80°C overnight, followed by thawing and homogenising with a motor-assisted pestle. 60μl of chloroform was added to the suspension, and mixed by vigorous shaking for 15 seconds. The mixture was centrifuged at 4°C at 12,000 x g for 15 minutes, and the upper aqueous layer was transferred into a fresh microcentrifuge tube. Total RNA was precipitated with 150μl of isopropanol at room temperature for 10 minutes, and pelleted by

centrifugation at 4°C at 12,000 x g for 10 minutes. The supernatant was removed, the pellet was washed with 70% ethanol, and resuspended in 17µl of RNase-free water. The sample was added with 2µl of 10x TURBO DNase Buffer (Ambion) and digested with 2 units of TURBO DNase at 37°C for 15 minutes. The reaction mixture was filled to 100µl with water, and then mixed with 100µl of acid phenol/chloroform/isoamyl alcohol (25/24/1, pH4.5) by vortexing, followed by centrifugation at 4°C at 12,000 x g for 10 minutes. The aqueous layer was collected, and extracted with 100µl of chloroform to remove residual phenol. The sample was precipitated with 10µl of 3M sodium acetate pH5.2 and 350µl of ethanol, at -20°C overnight. Following subsequent centrifugations and washings, the pellet was resuspended in 16µl of distilled water.

The RNA was quantified with the Quant-iT™ RNA Assay Kit (Invitrogen) and 4.5 µg was used in the 20µl *in vitro* transcription reaction with OligodT<sub>18</sub> primer, which binds to the poly-adenylated tails of mature mRNAs, and Superscript III reverse transcriptase (Invitrogen) with the following procedures:

| Reagents                   | Quantity     |
|----------------------------|--------------|
| 50µM OligodT <sub>18</sub> | 1µl          |
| 10mM dNTPs                 | 1µl          |
| Total RNA                  | 4.5µg        |
| RNase-free water           | Fill to 13µl |



|   |     |
|---|-----|
| Incubated at 65°C for 5 minutes, chilled on ice.  |     |
| 5x FS Buffer  | 4µl |
| 0.1M DTT  | 1µl |
| RNAseOUT RNAse inhibitor (40 U/µl)  | 1µl |
| Superscript III reverse transcriptase<br>(200 U/µl)   | 1µl |
| Incubated at 50°C for 90 minutes, and then at 85°C for 20 minutes. 1 unit of RNAseH (NEB) was added, followed by incubation at 37°C for 20 minutes. |     |

The first strand cDNA was quantified with the Quant-iT™ ssDNA Assay Kit (Invitrogen), and stored at -20°C. All regenerating samples were frozen at 72 hours post amputation.

#### 2.2.11 Cloning of *Smed\_Tert* cDNA and discovery of isoforms

TERT protein sequences from multiple species were aligned with the sexual *S.mediterranea* genome assembly using tBLASTn, and the highest homology was found on the negative strand in Contig v31.000666. Putative exons surrounding the site of the blast hit were predicted using Genscan (<http://genes.mit.edu/GENSCAN.html>) and two ESTs were mapped to the genomic region within the predicted transcript. EST accession number ec1.13248.001(Robb et al., 2008) locates on the 5' part

of the cDNA which appears to contain also a part of the 5' UTR, while PL08003A2F11(Zayas et al., 2005) was mapped to the 3' part of the cDNA with a part of the 3' UTR. A set of primers were designed to amplify nearly the full length of the ORF from the cDNA in both strains (forward: TERT\_F7; reverse: TERT\_R), the PCR conditions are listed as follows:

| Total reaction volume: 50µl          |                     | Cycling conditions |             |          |
|--------------------------------------|---------------------|--------------------|-------------|----------|
| Reagent                              | Final concentration | Step               | Temperature | Duration |
| 5x HF Buffer<br>(Finnzymes)          | 1x                  | 1                  | 98°C        | 2 min    |
| 2mM dNTP                             | 0.2mM               | 2                  | 98°C        | 12 sec   |
| Primer:<br>TERT_F7<br>(10µM)         | 300nM               | 3                  | 59°C        | 20 sec   |
| Primer:<br>TERT_R (10µM)             | 300nM               | 4                  | 72°C        | 90 sec   |
| cDNA                                 | 1ng/µl              | Repeat             | Step 2-4    | 36 Times |
| Phusion<br>polymerase<br>(Finnzymes) | 0.06U/µl            | 5                  | 72°C        | 5 min    |

|                 |              |  |  |
|-----------------|--------------|--|--|
| Deionised water | Fill to 50µl |  |  |
|-----------------|--------------|--|--|

The amplified products were cloned into pCR2.1 TOPO vectors, colonies were selected with 50µg/ml of kanamycin and blue-white screening in the presence of X-gal (20 mg/ml in DMSO). Positive colonies were randomly picked and inserts of different lengths were sequenced for the detection of isoforms. UTR sequence at both ends was confirmed by 3' and 5' RACE with the FirstChoice RLM-RACE Kit (Ambion) (see section 2.2.17 and 2.2.18).

### 2.2.12 Quantitative reverse transcription PCR

qRT-PCR was performed with the first strand cDNA described above using the Brilliant qPCR master mix (Agilent), all reactions were incubated at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, annealing temperature for 20 seconds, and 72°C for 20 seconds. The transcription level of an internal control gene *cystatin* was used in each sample to standardise the template concentration. Each experiment was done in triplicate, with a pool of tRNA from 5 worms per replicate. Primers for each reaction are listed as follows:

| Amplifying target             | Primers      | Annealing temperature | Primer Concentration (nM) |
|-------------------------------|--------------|-----------------------|---------------------------|
| All <i>Smed_Tert</i> isoforms | TERT_qPCR_F3 | 54°C                  | 300                       |
|                               | TERT_qPCR_R3 |                       | 300                       |

|                                       |                    |      |     |
|---------------------------------------|--------------------|------|-----|
|                                       |                    |      |     |
| Alt Region I +ve isoforms             | TERT_qPCR_AltI_F1  | 54°C | 450 |
|                                       | TERT_qPCR_AltI_R1  |      | 450 |
| Alt Region I –ve isoforms             | TERT_qPCR_AltI_F2  | 51°C | 200 |
|                                       | TERT_qPCR_AltI_R2  |      | 200 |
| Alt Region II +ve isoforms            | TERT_qPCR_AltII_F1 | 55°C | 300 |
|                                       | TERT_qPCR_AltII_R1 |      | 300 |
| <i>cystatin</i><br>(internal control) | Cys_qPCR_F         | 55°C | 300 |
|                                       | Cys_qPCR_R         |      | 300 |
| Category 1 cells                      | Smedwi-2_F         | 55°C | 300 |
|                                       | Smedwi-2_R         |      | 300 |
| Category 2 cells                      | NB.21.11e_F        | 55°C | 300 |
|                                       | NB.21.11e_R        |      | 300 |
| Category 3 cells                      | Agat1_F            | 56°C | 300 |

|  |         |  |     |
|--|---------|--|-----|
|  | Agat1_R |  | 300 |
|--|---------|--|-----|

### 2.2.13 Whole mount in-situ hybridisation of *Smed\_Tert* mRNA

The experiment was performed as previously described (Umesono et al., 1997) with modifications (Nogi and Levin, 2005, Gonzalez-Estevez et al., 2009).

#### 2.2.13.1 Synthesis of 5'-DIG labelled probe

5'-DIG labelled antisense probe was produced from the primary PCR product for double-stranded RNA synthesis described in section 2.2.1 (with the TERT\_F2/ TERT\_R primer set). A secondary PCR was done with the TERT\_F2/Uni\_T7\_3' primer set (using the same conditions as the primary PCR) to attach a T7 RNA polymerase binding site specifically on the 3' end of the primary PCR product. Single-stranded antisense probe was produced by *in vitro* transcription of the secondary PCR product, using the following procedures:

| Total reaction volume: 10µl      |                     | Procedure  |
|----------------------------------|---------------------|--|
| Reagent                          | Final concentration | Incubated at 37°C for 8.5 hours.   |
| 10x transcription buffer (Roche) | 1x                  | Added 2 units of TURBO DNase (Ambion), incubated at 37°C for 30 minutes. |
| 20mM rNTP                        | 1x                  | Filled to 35µl with water, and added with 5µl of 4M Lithium              |

|                                       |                    |  |
|---------------------------------------|--------------------|--|
|                                       |                    | chloride   |
| Secondary PCR product                 | 35ng/ $\mu$ l      | Precipitated with 150 $\mu$ l of EtOH at -20°C overnight.  |
| RNaseOUT RNase inhibitor (Invitrogen) | 1U/ $\mu$ l        | Centrifuged at 12,000 x g for 30 minutes, supernatant removed.   |
| T7 RNA polymerase (Roche)             | 4U/ $\mu$ l        | Pellet washed with 1ml of 70% EtOH, then resuspended in 20 $\mu$ l of resuspension buffer by heating at 65°C for 10 minutes. |
| RNase-free water                      | fill to 10 $\mu$ l | Quantified on a gel as described in section 2.2.1.   |

### *2.2.13.2 Fixation and bleaching of whole planarian samples*

Samples used in this experiment include intact, regenerating, and gamma-irradiated worms of both strains. All regenerating samples were cut into head, middle, and tail pieces, and allowed to regenerate for 72 hours.

Worms were killed with 2% hydrochloric acid in 5/8 Holtfreter's solution on ice for no more than 5 minutes, and then fixed with Carnoy solution (60% ethanol, 30% chloroform, 10% glacial acetic acid) for 2 hours on a shaker at 4°C. Samples were left in 100% methanol at -20°C for at least one hour, followed by bleaching with 6% hydrogen peroxide in methanol for approximately 20 hours under a cold lamp at room temperature.

### *2.2.13.3 Whole mount in situ hybridisation*

Bleached worms were hydrated using a series of 70%, 50%, and 30% ethanol in 5/8 Holtfreter's solution, each for 30 minutes at 4°C. The worms were permeabilised with PBSTx (0.1% TritonX-100/PBS) for 30 minutes at 4°C and then with 20µg/ml of Proteinase K in PBSTx for 6-12 minutes at 37°C (the duration of Proteinase K treatment was determined by the size of the samples). The worms were rinsed in 5/8 Holtfreter's solution, and fixed with 4% PFA in 5/8 Holtfreter's solution for one hour at 4°C, followed by washing in 5/8 Holtfreter's solution for one hour at 4°C. To remove any discolouration, the worms were incubated in 0.1M TEA pH7.6 for 15 minutes at room temperature; this step was repeated with fresh TEA, followed by adding 25µl of acetic anhydride to the TEA and shaking at room temperature for 15 minutes. This addition was repeated with another 25µl of acetic anhydride and the samples were incubated for 15 minutes at room temperature. The worms were washed in PBS for 15 minutes at room temperature and incubated in 500µl of pre-heated prehybridisation buffer at 56°C for at least one hour on a rocker inside a Techne Hybridiser HB-1D oven. The 5'-DIG-labelled probe were added to 400µl of hybridisation buffer to a final concentration of 0.5 ng/µl, and then denatured at 72°C for 10 minutes and chilled on ice for 2 minutes. The prehybridisation buffer was replaced by the hybridisation buffer containing the probe, followed by hybridisation at 56°C for 20 hours.

The post hybridisation solutions (PostHyb1, PostHyb2, PostHyb3, PostHyb4, PostHyb5, PostHyb6, Buffer I, and Buffer II) were freshly made, and PostHyb solutions 1-6 were pre-warmed at 56°C before use. The samples were rinsed three times with PostHyb1 solution, transferred to a 6 well plate, and washed in 3ml of PostHyb1 solution for 15 minutes at 56°C. The

samples were subsequently washed in 3ml of PostHyb 2-4 for 10 minutes each at 56°C, and then twice in 3ml of PostHyb5 and PostHyb6 for 30 minutes at 56°C. The worms were washed twice in Buffer I for 10 minutes at room temperature, and then transferred to a 24 well plate and blocked with Buffer II for 1 hour at room temperature. The samples were incubated with 500µl of 1:2000 anti-DIG-AP antibody (Roche) in Buffer II for 3 hours at room temperature, and then rinsed twice in Buffer I, incubated twice in Buffer I for 10 minutes, and incubated overnight in Buffer I at 4°C.

Following the incubation, the samples were washed twice in Buffer I for 10 minutes at room temperature, and then incubated in the TMN solution in dark for 5 minutes at room temperature. Staining was developed with NBT/BCIP (Roche) in TMN (20µl of NBT/BCIP solution per 1ml of TMN) in dark at room temperature. The development was stopped by washing in 1x PBS and fixed in 4% PFA in PBS for one hour at room temperature. The samples were rinsed with PBS and washed with a series of ethanol in PBS (30%, 50%, 70% for 5 minutes, 100% for 30 minutes, and then 70%, 50%, 30% for 5 minutes) to reduce the background. The worms were washed in PBS and mounted onto glass slides in 70% glycerol/PBS and observed on a Zeiss Discovery V8 stereomicroscope, images were taken using an Axio Cam MRC camera.

Two irradiation control probes were hybridised separately in this experiment, these include a neoblast-specific marker *Smed-HistoneH2B* at a working concentration of 0.2 ng/µl, and an end-differentiated neuronal marker *Smed-GluR* at 0.18 ng/µl.



#### 2.2.14 Anti-phosphorylated histone H3 ser10

##### immunohistochemistry

The worms were fixed and bleached as described in section 2.2.13, and then washed in a series of 75%, 50%, and 25% methanol in PBSTx (0.3% TritonX-100 in PBS) for 10 minutes each at room temperature. The worms were washed twice in PBSTx for 10 minutes before they were blocked with 1% BSA in PBSTx for 2 hours at room temperature. The samples were incubated with an anti-phosphorylated histone H3 serine 10 rabbit monoclonal primary antibody (Upstate Millipore, diluted 1:600 in 1% BSA/PBSTx) for 43 hours at 4°C. The samples were washed in 1ml of PBSTx for 8 hours at room temperature, with replacement of PBSTx with fresh solution every 30 minutes. The samples were then blocked with 1% BSA in PBST for 1 hour at room temperature, and incubated with an Alexa Fluor 568-conjugated goat anti-rabbit secondary antibody (Molecular Probes, diluted 1:1000 in 1% BSA/PBSTx) in dark at 4°C for 14-16 hours. The samples were washed for 4 hours in PBSTx in dark at room temperature, with the solution replenished every 30 minutes, followed by fixing overnight with 4% PFA in PBS in dark at 4°C. The samples were washed twice in PBS for 10 minutes at room temperature and mounted in 1:2 Slowfade antifade (Invitrogen) diluted in 50% glycerol/PBS.

Staining was visualised on a Leica MZ16F fluorescent stereomicroscope with a Leica DFC 300Fx camera (Leica Lasertechnik, Heidelberg), positive cells were counted using the ImageJ software.

### 2.2.15 Metaphase chromosome spread

This method was modified from Lamatsch et al, 1998 (Lamatsch et al., 1998). Planarian samples (6 worms per sample) were amputated and treated with 0.065% colchicine at 56 hours post cut for 16 hours. The regenerating tissues, including the post-blastema regions, were cut off from the rest of the body and hypotonically shocked with 1M sodium citrate for 20 minutes at room temperature. The tissues were then fixed in Carnoy solution (ethanol: acetic acid 3:1, differs from the one used for *in-situ* hybridisation) at 4°C for 30 minutes, followed by cell dissociation with 55µl of 45% acetic acid at room temperature for 16 hours. The cell suspensions were centrifuged at 200g for 5 minutes, and the supernatant was dropped onto chilled slides (pre-treated by soaking in chilled 70% ethanol and polished with paper towel before use). The slides were immediately covered with slips and snap-frozen on dry ice, the coverslips were removed before the slides thawed. The slides were allowed to dry on the bench, and then soaked in Carnoy solution for 5 minutes, followed by air-dry and staining with DAPI in PBS in dark for 20 minutes. The DAPI solution was rinsed off with deionised water and the slides were mounted in PBS and observed with a Nikon eclipse 80i fluorescent compound microscope. Images were taken with a Hamamatsu ORCA-ER digital camera.

### 2.2.16 3' Rapid amplification of cDNA ends (RACE) of *Smed\_Tert* and *Smed\_Pot1*

The template cDNA used in 3' RACE was synthesised with the same procedure as the first strand cDNA described in section 2.2.10. The attached OligodT<sub>18</sub>, adapted from the OligodT<sub>12</sub> of the FirstChoice RLM-RACE kit (Ambion), at the end of each transcript provides binding sites for

a nested reverse PCR primer set, which when paired with a set of gene-specific nested primers, can amplify the region between currently known sequence of the gene and the 3' end of the transcript. PCR reactions for *Smed\_Tert* and *Smed\_Pot1* are listed as follows:

#### Outer PCR

|   |                     |                    |             |      |          |
|---|---------------------|--------------------|-------------|------|----------|
| Total reaction volume: 50μl                         |                     | Cycling conditions |             |      |          |
| Reagent   | Final concentration | Step               | Temperature |      | Duration |
|   |                     |                    | Tert        | Pot1 |          |
| 5x HF Buffer<br>(Finnzymes)                         | 1x                  | 1                  | 98°C        | 98°C | 2 min    |
| 2mM dNTP  | 0.2mM               | 2                  | 98°C        | 98°C | 12 sec   |
| Primer:<br>TERT_3'Out_F /<br>POT1_3'Out_F<br>(10μM) | 300nM               | 3                  | 58°C        | 58°C | 20 sec   |
| Primer:<br>3'Out_R (10μM)                           | 300nM               | 4                  | 72°C        | 72°C | 60 sec   |
| cDNA  | 1ng/μl              | Repeat             | Step 2-4    |      | 34 Times |
| Phusion<br>polymerase<br>(Finnzymes)                | 0.06U/μl            | 5                  | 72°C        | 72°C | 2 min    |

|                 |              |  |  |  |  |
|-----------------|--------------|--|--|--|--|
|                 |              |  |  |  |  |
| Deionised water | Fill to 50µl |  |  |  |  |

### Inner PCR

| Total reaction volume: 50µl                         |                     | Cycling conditions |             |             |          |
|---|---------------------|--------------------|-------------|-------------|----------|
| Reagent   | Final concentration | Step               | Temperature |             | Duration |
|   |                     |                    | <i>Tert</i> | <i>Pot1</i> |          |
| 5x HF Buffer<br>(Finnzymes)                         | 1x                  | 1                  | 98°C        | 98°C        | 2 min    |
| 2mM dNTP  | 0.2mM               | 2                  | 98°C        | 98°C        | 12 sec   |
| Primer:<br>TERT_3'Inn_F /<br>POT1_3'Inn_F<br>(10µM) | 300nM               | 3                  | 58°C        | 58°C        | 20 sec   |
| Primer:<br>3'Inn_R (10µM)                           | 300nM               | 4                  | 72°C        | 72°C        | 60 sec   |
| Outer PCR<br>product                                | 1µl total           | Repeat             | Step 2-4    |             | 34 Times |
| Phusion<br>polymerase                               | 0.06U/µl            | 5                  | 72°C        | 72°C        | 2 min    |

|                 |              |  |  |  |  |
|-----------------|--------------|--|--|--|--|
| (Finnzymes)     |              |  |  |  |  |
| Deionised water | Fill to 50µl |  |  |  |  |

The inner PCR products were gel-extracted, cloned into pCR 2.1 TOPO plasmids (Invitrogen), and confirmed by sequencing.

### 2.2.17 5' RACE of *Smed\_Tert* and *Smed\_Pot1*

Differed from the 3' RACE, the 5' RACE requires removal of phosphates and the cap structure at the 5' end of each mRNA, followed by ligation of the 5' RACE adaptor to the end, which provides recognition sites for nested forward primers. Subsequent PCR amplifications with these primers, together with gene-specific reverse nested primers, allow the identification of unknown sequences between a fragment of a gene and its 5' end.

Reactions were performed with reagents supplied in the FirstChoice RLM-RACE kit (Ambion). Total RNA was extracted from the worms as described in section 2.2.10. 1µg of the total RNA was treated with 2µl of Calf Intestine alkaline Phosphatase (CIP) in presence of 1x CIP Buffer in a 20µl reaction. The mixture was incubated at 37°C for one hour, after which 15µl of ammonium acetate stop solution was added and the mixture was filled to 150µl with RNase-free water. The RNA was purified with a phenol-chloroform extraction, and precipitated with an equal volume of isopropanol on ice for 10 minutes. After centrifugation and washing with 70% ethanol, the RNA was resuspended in 4µl of 1x Tobacco Acid Pyrophosphatase (TAP) Buffer, and incubated with 1µl of TAP at 37°C for one hour. The treated RNA was ligated with 300ng of the 5' RACE RNA

Adaptor using 2µl of T4 RNA ligase in presence of 1X Ligase Buffer, in a 10µl reaction at 37°C for one hour.

Reverse transcription of the ligated total RNA was done using the SuperScript III reverse transcriptase (Invitrogen), with the following procedures:

| Reagents   | Quantity     |
|--|--------------|
| 50µM Random Decamers (from the RACE kit)         | 1µl          |
| 10mM dNTPs                                       | 1µl          |
| Ligated total RNA                                | 2µl          |
| RNAse-free distilled water                       | Fill to 13µl |
| Incubated at 65°C for 5 minutes, chilled on ice. |              |
| 5x FS Buffer                                     | 4µl          |
| 0.1M DTT   | 1µl          |
| RNAseOUT RNAse inhibitor (40 U/µl)               | 1µl          |
| Superscript III reverse transcriptase (200 U/µl) | 1µl          |

|   |  |
|---|--|
|   |  |
| Incubated at 50°C for 60 minutes, and then at 85°C for 20 minutes. 1 unit of RNaseH (NEB) was added, followed by incubation at 37°C for 20 minutes. |  |

The cDNA was not quantified, and was used directly for the outer RACE PCR. The PCR reactions are listed as follows:

#### Outer PCR

|   |                     |                    |             |      |          |
|---|---------------------|--------------------|-------------|------|----------|
| Total reaction volume: 50µl                         |                     | Cycling conditions |             |      |          |
| Reagent   | Final concentration | Step               | Temperature |      | Duration |
|   |                     |                    | Tert        | Pot1 |          |
| 5x HF Buffer (Finnzymes)                            | 1x                  | 1                  | 98°C        | 98°C | 2 min    |
| 2mM dNTP  | 0.2mM               | 2                  | 98°C        | 98°C | 12 sec   |
| Primer:<br>TERT_5'Out_R /<br>POT1_5'Out_R<br>(10µM) | 300nM               | 3                  | 60°C        | 60°C | 20 sec   |
| Primer:<br>5'Out_F (10µM)                           | 300nM               | 4                  | 72°C        | 72°C | 60 sec   |
| cDNA  | 1µl total           | Repeat             | Step 2-4    |      | 34 Times |

|                                      |              |  |   |      |      |       |
|--------------------------------------|--------------|--|---|------|------|-------|
|                                      |              |  |   |      |      |       |
| Phusion<br>polymerase<br>(Finnzymes) | 0.06U/μl     |  | 5 | 72°C | 72°C | 2 min |
| Deionised water                      | Fill to 50μl |  |   |      |      |       |

### Inner PCR

| Total reaction volume: 50μl                         |                     | <b>Cycling conditions</b> |             |             |          |
|---|---------------------|---------------------------|-------------|-------------|----------|
| Reagent   | Final concentration | Step                      | Temperature |             | Duration |
|   |                     |                           | <i>Tert</i> | <i>Pot1</i> |          |
| 5x HF Buffer (Finnzymes)                            | 1x                  | 1                         | 98°C        | 98°C        | 2 min    |
| 2mM dNTP  | 0.2mM               | 2                         | 98°C        | 98°C        | 12 sec   |
| Primer:<br>TERT_5'Inn_R /<br>POT1_5'Inn_R<br>(10μM) | 300nM               | 3                         | 58°C        | 62°C        | 20 sec   |
| Primer:<br>5'Inn_F (10μM)                           | 300nM               | 4                         | 72°C        | 72°C        | 40 sec   |
| Outer PCR   | 1μl total           | Repeat                    | Step 2-4    |             | 34 Times |



|                                      |              |  |   |      |      |       |
|--------------------------------------|--------------|--|---|------|------|-------|
| product                              |              |  |   |      |      |       |
| Phusion<br>polymerase<br>(Finnzymes) | 0.06U/μl     |  | 5 | 72°C | 72°C | 2 min |
| Deionised water                      | Fill to 50μl |  |   |      |      |       |

The inner PCR products were gel-extracted, cloned into pCR 4 TOPO plasmids (Invitrogen), and confirmed by sequencing.

## CHAPTER 3: TELOMERE LENGTH AND ITS MAINTENANCE IN *S.mediterranea*

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### 3.1 Introduction

#### 3.1.1 Finding the telomeric repeat unit in *S. mediterranea*

The presence of (TTAGGG)<sub>n</sub> terminal sequence in *Polycelis tenuis* (Joffe et al., 1996) has suggested that the telomere of the closely related *S. mediterranea* also consists of the same tandem repeats. This hypothesis is supported by large tandem (TTAGGG)<sub>n</sub> repeat regions located at one end of several contigs or trace reads in the *S. mediterranea* genome. Table 3.1.1 contains a list of currently identified contigs and reads which contain possible telomeric sequences.

| Type       | Name             | Possible telomeric region |
|------------|------------------|---------------------------|
| Trace read | ujm06g02.b1      | 263 – 643 bp              |
| Trace read | uki42d10.b1      | 176 – 556 bp              |
| Trace read | SAAE-adi73e04.g1 | 76 – 323 bp               |
| Trace read | SAAE-agm29d05.g1 | 71 – 330 bp               |
| Trace read | SAAA-ajg75g07.b1 | 29 – 254 bp               |
| Trace read | SAAA-ajg80e01.b1 | 33 – 246 bp               |
| Contig     | v31.000477       | 1 – 175 bp                |

**Table 3.1.1** – Possible telomeric regions in *S. mediterranea* genomic contigs and trace reads.

### 3.1.2 Confirming telomeric sequence by the Terminal Restriction Fragment (TRF) analysis

Experiments were designed to measure the length of (TTAGGG) $n$  repeats in the genome and to confirm that these repeats belong to chromosomal ends. A restriction endonuclease which recognises and cuts a specific pattern of six nucleotides (ie. a "6-cutter") should cut on average every 4096bp ( $4^6$ , calculated from the probability of a specific recognition site sequence arisen by random chance). Assuming every additional 6-cutter enzyme splits the average fragment size by a half, treating gDNA with a combination of five 6-cutters will theoretically produce fragments with an average size of 256bp ( $4^6$  divided by  $2^{(5-1)}$ ). The selected enzymes do not cut (TTAGGG) $n$  repeats, and therefore the size of the restriction fragments visualised by the (TTAGGG) $n$  probe in southern hybridisation should be similar to the real size of these repeat sequences (Harley et al., 1990). In order to confirm that the signal on the blot was not produced by large internal (TTAGGG) $n$  repeats, the gDNA was treated with the Bal-31 exonuclease (Blackburn and Challoner, 1984), which degrades both 3' and 5' termini of duplex DNA, prior to the endonuclease digestion. Telomeric sequences are sensitive to the degradation while internal repeats should not be affected by this treatment. The use of 6-cutters allows detection of known internal sequences which can be used as a control for the effect of Bal-31 cleavage. In contrast to the "4-cutters" which severely fragments internal sequences, the 6-cutters were selected to produce a 2787bp band of *nanos* gene which should not be sensitive to Bal-31 treatment.

### 3.1.3 Telomere length dynamics in sexual and asexual strains of *S. mediterranea*

In the absence of reproductive cells, the long term survivability of an asexual lineage relies, in theory, on a somatic stem cell population which

has an indefinite proliferative life-span. Since telomere integrity is essential for the viability of a cell, it can be hypothesised that the telomere lengths of the stem cells in asexual worms are properly protected from erosion during cell cycling, in order to ensure the proliferative capacity and genome stability of the cells over an evolutionary timescale. The maintenance is likely to be achieved by telomerase due to its common presence across eukaryotic kingdoms. The presence of the enzyme is known to be linked to proliferative cells in normal non-immortalised somatic human cells (Masutomi et al., 2003), and previous work has also shown telomere extension events, as well as increase in TERT expression and telomerase activity in S phase cells in human and yeast (Masutomi et al., 2003, Wellinger et al., 1993). Therefore telomere maintenance in the stem cell population in *S. mediterranea* is likely to be linked to events involving massive cell divisions. Previous studies have shown peaks in proliferative cells in regenerating planarians, with the first mitotic maximum occurring at approximately 6 hours post amputation and a higher second peak occurring at 2-3 days post cut (Baguñà, 1976b, Saló and Baguñà, 1984). The rapid cell divisions during regeneration could cause substantial telomere erosion unless a proper telomere maintenance mechanism is present. Since regeneration plays an essential part of asexual reproduction in *S. mediterranea*, mechanisms should be present to counteract the telomere erosion during this reproductive event. If telomerase is involved in the maintenance mechanism, it should be functional during S phase in cycling cells. Therefore telomere extension events should be concentrated during regeneration, when massive cell cycling occurs. It is likely that telomeres of an asexual worm are periodically replenished in its life cycle, through fission and subsequent regeneration. This theory is based on the fact that no functional germline is

present in asexual worms (Saló, 2006), and somatic stem cells therefore should possess the immortal property of germ cells.

The discovery of nanos positive primordial germ cells in the asexual strain (Sato et al., 2006, Wang et al., 2007b, Handberg-Thorsager and Salo, 2007) suggests an alternative model, in which the asexual worms are facultatively sexual, and telomeres are maintained in the primordial germ cells, which can give rise to somatic stem cells with replenished telomeres following fertilisation or parthenogenesis. Lack of reproductive organs in asexual worms indicates that if this mechanism exists, no genetic material is exchanged between strains, and fertilisation is confined within hermaphroditic individuals.

The sexual strain of *S. mediterranea* does not reproduce by fission/regeneration, and possesses a complete germline reproductive system. Therefore it is uncertain whether a well maintained somatic telomere is required for long-term survivability of the population. Although the life span of sexual *S. mediterranea* has not been previously studied, early work in various sexually reproducing relative triclad species have suggested a limited life expectancy in the sexual worms (Child, 1914, Haranghy and Balázs, 1964), a recent demographic study in sexual marine platyhelminthes has also seen a limited life span (Mouton et al., 2009). However sexual worms are also capable of clonal amplification in the laboratory by amputation, which suggests a stem cell population with high proliferative and self-renewal capacity. It is likely that the sexual strain also shares the same somatic telomere replenishing mechanism with the asexual worms after amputation, since the regenerated fragments are *de facto* juvenile worms with no mature germ cells and gonadopores, and

their somatic age may need to be reset to match their reproductive age. While for sexual worms without injuries, the somatic cells may age, limiting the life span of parents and giving space for offspring which contain more genetic variation. Since sexual worms do not self-induce injuries as the asexuals, their telomere maintenance should normally be concentrated in the germline instead of the soma. Telomeres in other multicellular organisms are well maintained in the germline (De Lange et al., 1990, Allsopp et al., 1992, Fiset and Chabot, 2001), consistent with the fact that telomerase is highly expressed during gametogenesis (Mantell and Greider, 1994, Yashima et al., 1998) and/or embryogenesis (Schaetzlein et al., 2004). It is likely that an un-injured homeostatic sexual worm uses the same telomere maintenance strategy as other sexual organisms (high in germline and low the soma), and after body fragmentation the telomere of the soma is reset.

In this project, the telomere length of both strains was measured at different stages of the life cycle. Additionally, the effect of regeneration on telomere length in both strains was also investigated. To obtain the size of telomeres with more precision, the gDNA was digested with a mixture of three 4-cutters, of which the cutting sites are theoretically closer to the tandem repeats than those of the 6-cutters due to their frequent cutting nature, which in addition to the smaller average internal fragments (64 bp), produce purer terminal restriction fragments after FIGE separation.

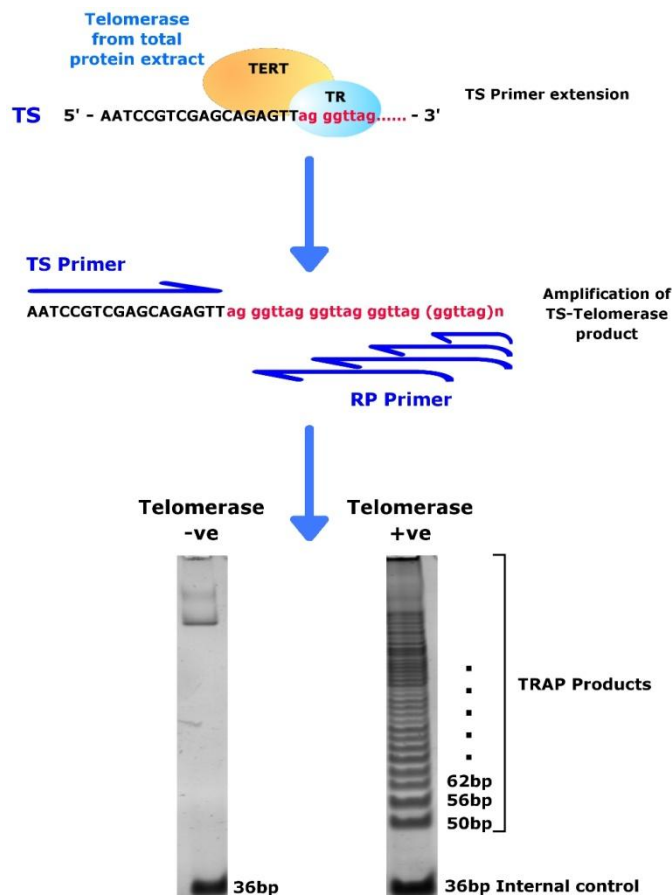
In addition to the TRF analysis, a qPCR based method (Cawthon, 2002) was used to measure the copy number of telomeric repeat sequence in the genome. Primers used for amplifying telomeric sequence usually form staggered dimers due to the tandem repeat nature of this region. The

modified oligonucleotides used in this technique were designed to have a mismatch to the genomic telomeric sequence every six bases but perfect complementarity at the last five 3' bases. This design results in a mismatched 3' end when the two primers are hybridised, preventing the formation of dimer products. The first six bases at the 5' end of both primers do not hybridise to the genomic DNA, therefore the amplified PCR product will not act as primers in later cycles. When the qPCR reaction is finished at the log phase, the relative telomere length of each sample can be determined as a factor by which the sample differs from a reference sample in the ratio of the telomeric copy number to the copy number of a single gene (ie. the T/S value) (Cawthon, 2002). This technique was applied to confirm the difference in the telomere length between the strains.

#### **3.1.4 Telomere maintenance activities in both strains of *S. mediterranea***

Presence of telomerase enzyme activity was assayed with the Telomere Repeat Amplification Protocol (TRAP) (Kim et al., 1994). This method is based on incubation of a synthetic substrate of telomerase, TS primer, with the total protein extract, followed by a PCR amplification to detect the elongation of the TS primer by the telomerase present in the total protein. In the presence of telomerase activity, PCR amplification with the TS and RP primer set of the kit produces a ladder of products with 6 base increments, starting at 50bp. An internal positive control for the PCR reaction (primer K1 and template TSK1), which produces a 36bp product, is included in the PCR reaction mix to identify false-negative results caused by Taq inhibitors (illustrated in Figure 3.1.1). The activity was compared between intact and regenerating worm samples in both strains. The link

between telomerase activity and proliferative cells was also examined by TRAP assay with worms treated with 100 Gy of gamma irradiation, which was previously shown to remove cells with proliferative capacity in planarians (Bardeen and Baetjer, 1904).



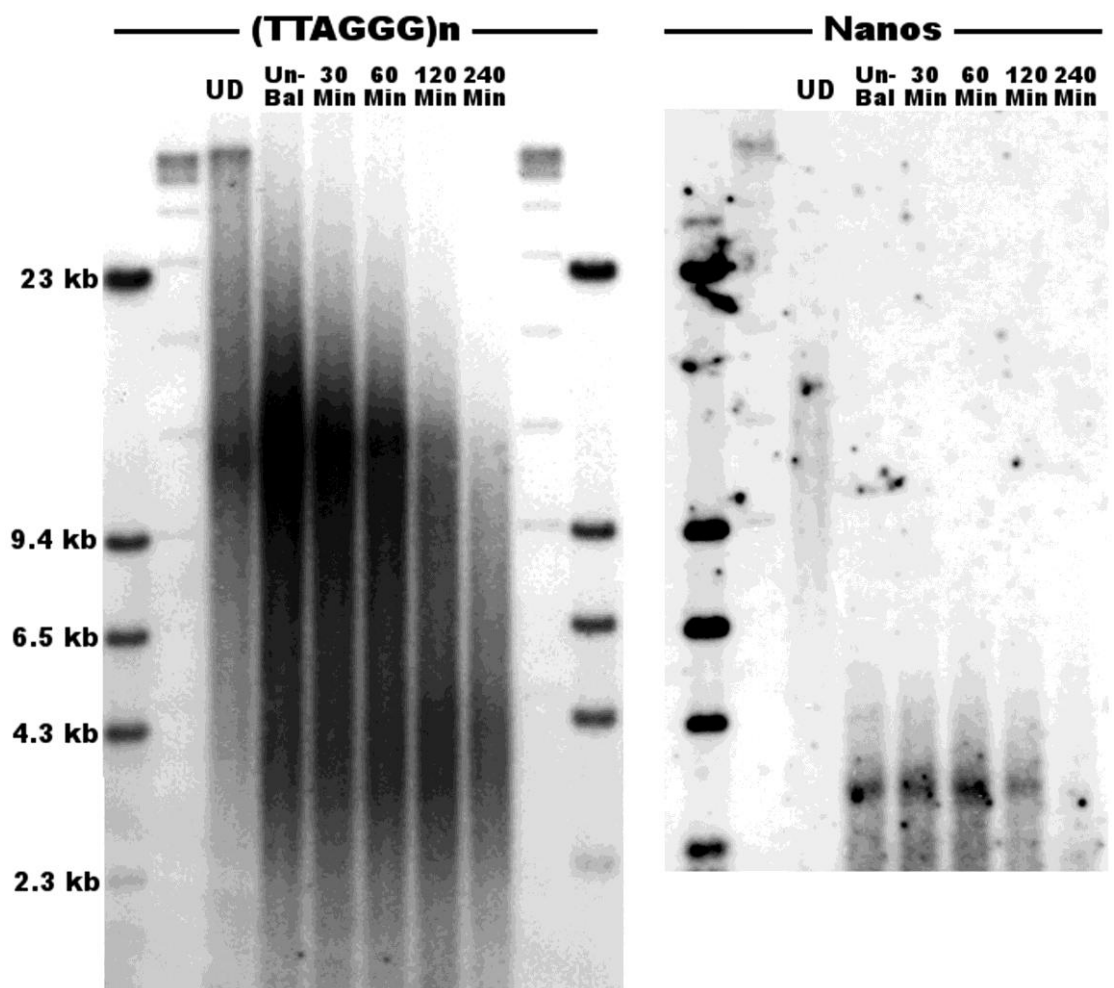
**Figure 3.1.1 - Schematic of the telomere repeat amplification protocol (TRAP) assay.** Incubation of TS primer with total protein extract causes the extension of the primer if telomerase is present. The extended TS primer can be detected by PCR amplification using TS and RP primer set, which gives a ladder of products with increments corresponding to the size of a tandem repeat unit (ie. 6bp for (TTAGGG)<sub>n</sub>).



## 3.2 Results

### 3.2.1 Confirmation of tandem repeat sequence at chromosome ends

The (TTAGGG)<sub>n</sub> signals on the blot was sensitive to the treatment with Bal-31 exonuclease. The mean sizes of the signals, defined as  $\Sigma(\text{ODi})/\Sigma(\text{ODi/Li})$  (see section 2.2.5), in samples treated with Bal-31 for various durations are listed in Table 3.2.1. Decrease in both the top and average sizes of the signals were observed as the duration of the treatment increased. In contrast, the band produced by the *nanos* probe stayed at a constant size of 2787bp (see Figure 3.2.1).



**Figure 3.2.1 – Bal-31 digestion of sexual gDNA followed by 6-cutter TRF analysis.** The effect of the exonuclease on telomeric repeat sequence (TTAGGG)<sub>n</sub> was compared to its effect on a gene sequence located internally (Nanos). UD: untreated gDNA; Un-Bal: gDNA digested with the 6-cutters without prior treatment with Bal-31; 30-240 Min: various durations of Bal-31 treatment.

| Duration of Bal-31 treatment | Mean signal length (kb) |
|------------------------------|-------------------------|
| 0 minute                     | 9.73                    |
| 30 minutes                   | 8.58                    |
| 60 minutes                   | 7.86                    |
| 120 minutes                  | 6.07                    |
| 240 minutes                  | 4.48                    |

**Table 3.2.1 – Mean sizes of (TTAGGG)<sub>n</sub> signals in Bal-31 treated samples.**

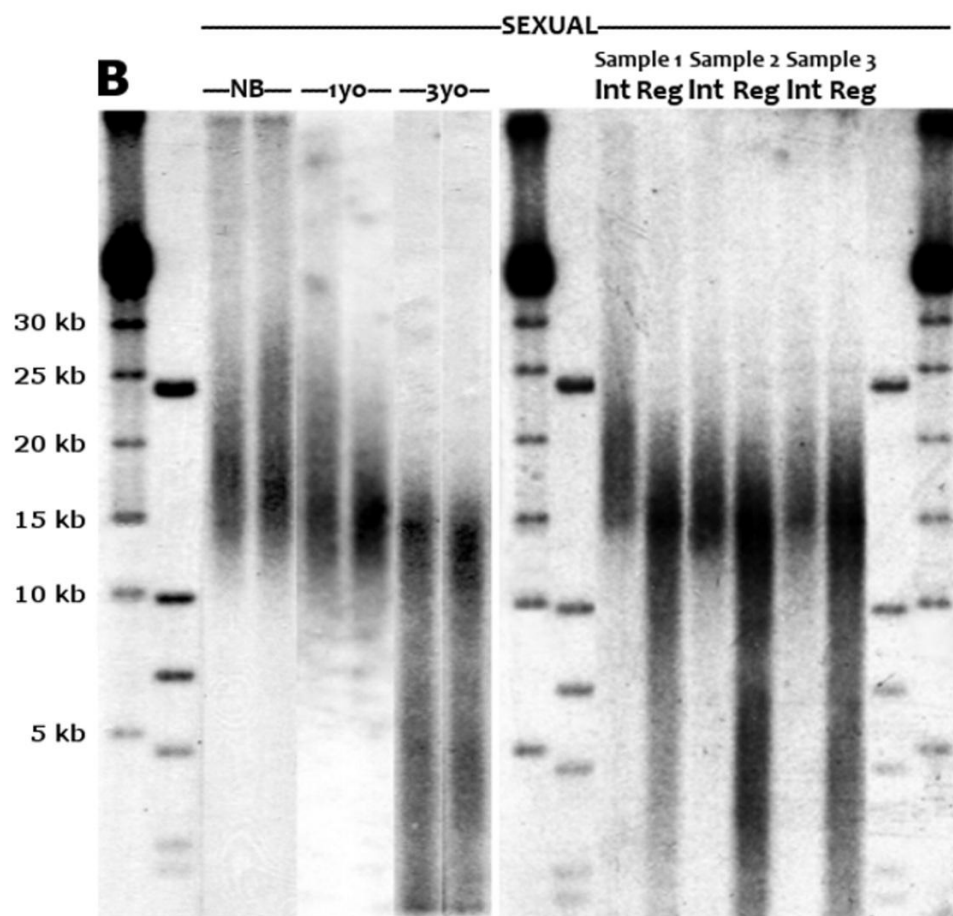
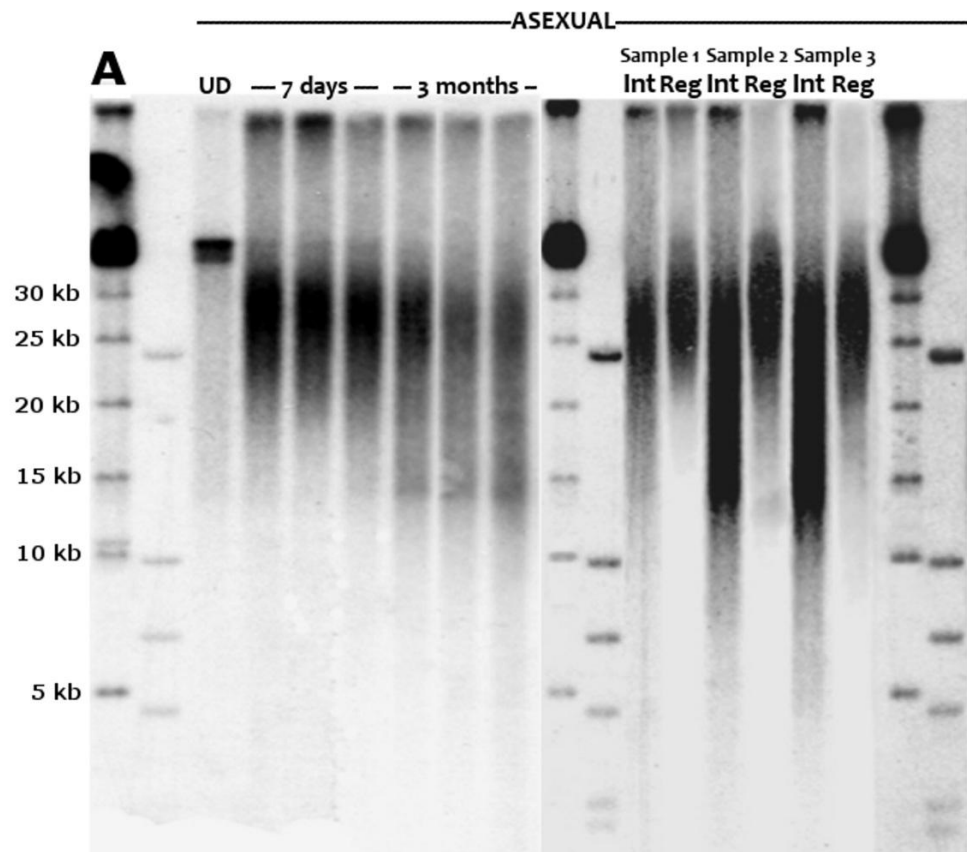
### 3.2.2 Telomere length dynamics in asexual and sexual strains of *S. mediterranea*

The telomeres in the asexual strain (see Figure 3.2.2a) range from 10kb to 35kb depending on the period of time since last fission or induced regeneration event. Samples extracted on the 7<sup>th</sup> day since last fission had a length distribution between 20 and 35kb, with a mean at 28.03kb and a standard deviation (s.d.) of the mean at 0.71kb (n=10). This is significantly longer (t-test, two tailed,  $p=0.0199$ ) than telomeres in samples extracted after 3 months post fission, which had a length distribution between 10 and 35kb, with a mean at 25.98kb and an s.d. at 0.60kb (n=10). Following three consecutive artificially induced regenerations in samples which had not undergone fission for between 3 and 6 months, the telomere length distributes between 20 and 40 kb (mean=26.93kb, s.d.=1.48kb, n=10), which is significantly longer (t-test, two tailed,  $p=0.0335$ ) than that of the same individual samples before regeneration (10 – 31kb, mean=22.67kb, s.d.=1.75kb, n=10).

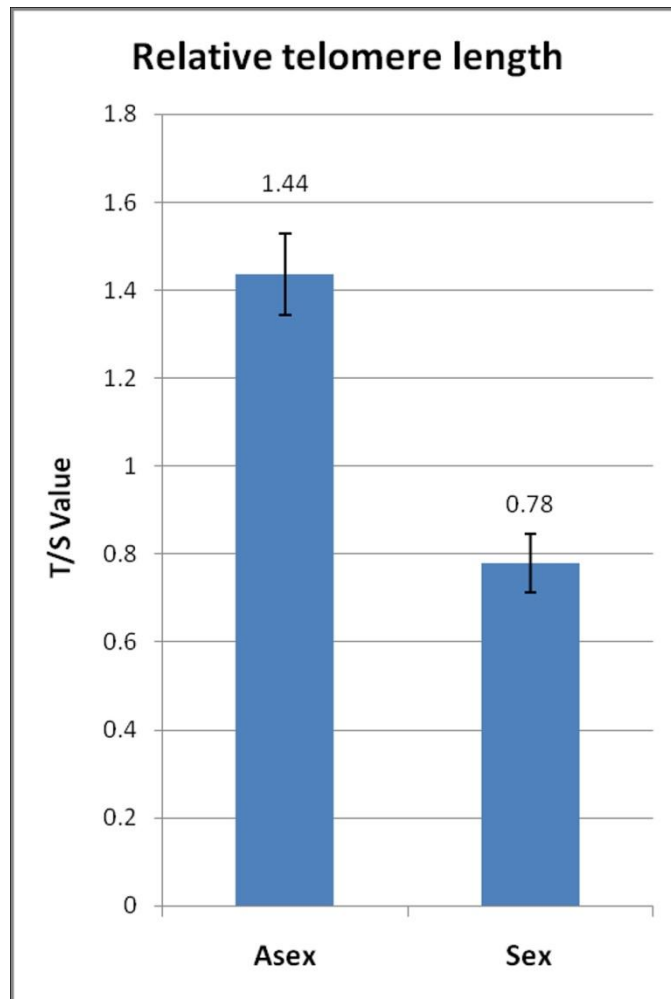
Telomere length in the sexual strain (see Figure 3.2.2b) was similar to previous findings in human and mice, with a decrease as a function of the age of the individuals; and the difference between individual worms was higher than that in the asexuals. Using sexual worms which were allowed to grow without events of induced regeneration, it was found that despite the heterogeneity between individuals, older worms have consistently shorter telomeres than younger samples. New-born hatchlings had a mean length at 21.20kb (s.d.=1.67kb, n=7), while the 1-year-old samples had a mean at 17.25kb (s.d.=2.03kb, n=7) and the 3-year-old samples at 11.06kb (s.d.=1.40kb, n=6). The lower ends of the distribution in the 3-year-old samples were shorter than the 2kb limit of the FIGE program, and

therefore the actual mean can be shorter than that calculated from the blot. In contrast to the telomere reset in the asexuals, induced regeneration had an opposite effect on the telomere length in sexual worms. Samples after three consecutive regeneration events had a telomere length between 2 – 20kb (mean=11.58kb, s.d.=1.49kb, n=10), which is significantly shorter (t-test, two tailed,  $p=0.00679$ ) than that of the same individuals before regeneration (13 – 22kb, mean=17.55kb, s.d.=1.33kb, n=10). All asexual and sexual samples regenerated eyes and pharynx by Day 7 post amputation, the rate of regeneration is ubiquitous among the samples and does not show correlation with the telomere length.

The relative telomere lengths in both strains measured by the qPCR technique were consistent with the results from the TRF analysis. After standardising with the single copy *NB.21.11e* gene, the telomeric copy number of the newly regenerated asexual fission sample was 1.44 times greater (three replicates of a batch of 5 worms, s.d.=0.0921) than that of the reference sample, which is an asexual worm grown for 3 months since last fission. In contrast, the 1-year-old sexual sample had a telomere copy number 78% of the reference (three replicates of a batch of 5 worms, s.d.=0.0655).



**Figure 3.2.2 – Strain-specific telomere length dynamics in *S. mediterranea*.** **(A)** Reset of telomere length in the asexual strain after fission and induced regeneration. UD: Undigested gDNA; The “7 days” samples represent worms newly regenerated from fission, while the “3 months” represent samples with no recent fission events; Int: Intact worm samples; Reg: Samples after 3 consecutive regeneration events. **(B)** Telomere erosions with age and after regeneration in the sexual strain. NB: New-born hatchlings; 1yo: 1 year old; 3yo: 3 year old; Int: Intact worm samples; Reg: Samples after 3 consecutive regeneration events.



**Figure 3.2.3 – Relative telomere length measured by qPCR.** The copy number of telomere repeats in newly fissioned asexual *S. mediterranea* samples was compared with that in 1-year-old sexual samples. An asexual sample extracted at three months since last fission event was used as the standard reference. Three individual samples were included in each strain.

### 3.2.3 Presence of telomere maintenance activity in *S. mediterranea* proteome

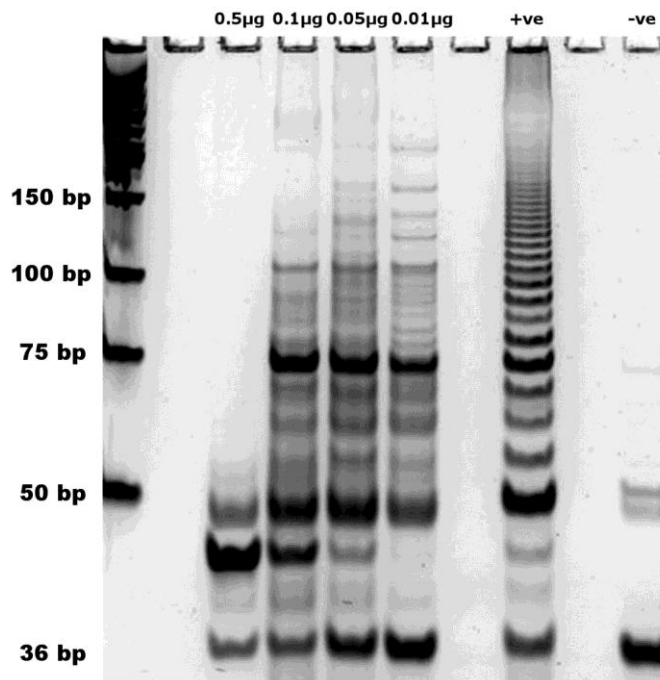
Several modifications from the manufacturer's protocol for the TRAP assay were required for optimal results in *S. mediterranea*. The incubation step was prolonged to 45 minutes from the recommended 30 minutes. As shown in Figure 3.2.4, incubation with more than 0.05µg of total protein produced an extra band, of which the intensity increased with the amount of total protein. Bands for the extended TS products also diminished as the total protein increased, indicating the presence of a TRAP inhibitor in the total protein. This inhibitory effect can be removed by a phenol-chloroform extraction step between the TS incubation and the PCR amplification, suggesting that the inhibitor targets Taq polymerase instead of telomerase. The duration of the elongation step of the PCR reaction was changed to 90 seconds, for a total of 35 cycles. The TRAP activity appeared to be sensitive to RNase, since reactions in the absence of RNase inhibitor failed to produce elongated products. Several brands of the RNase inhibitors were tested, including the RNaseOUT (Invitrogen), Protector (Roche), RNasin and RNasin Plus (Promega), it was found that the RNasin Plus provided the best protection against the RNase present in the total protein extract. Previous work of Ruman Rahman (unpublished) favoured the use of Roche Taq polymerase, which also produced better results in this project than the Bioline equivalent.

The TRAP assay detected the elongation of the TS primer after incubating with the total protein extract of *S. mediterranea*. The elongation occurred in a similar manner to the HeLa cell extract, a telomerase positive sample used as a control, which adds six bases to the substrate progressively. Differences in the activity could be observed between intact and

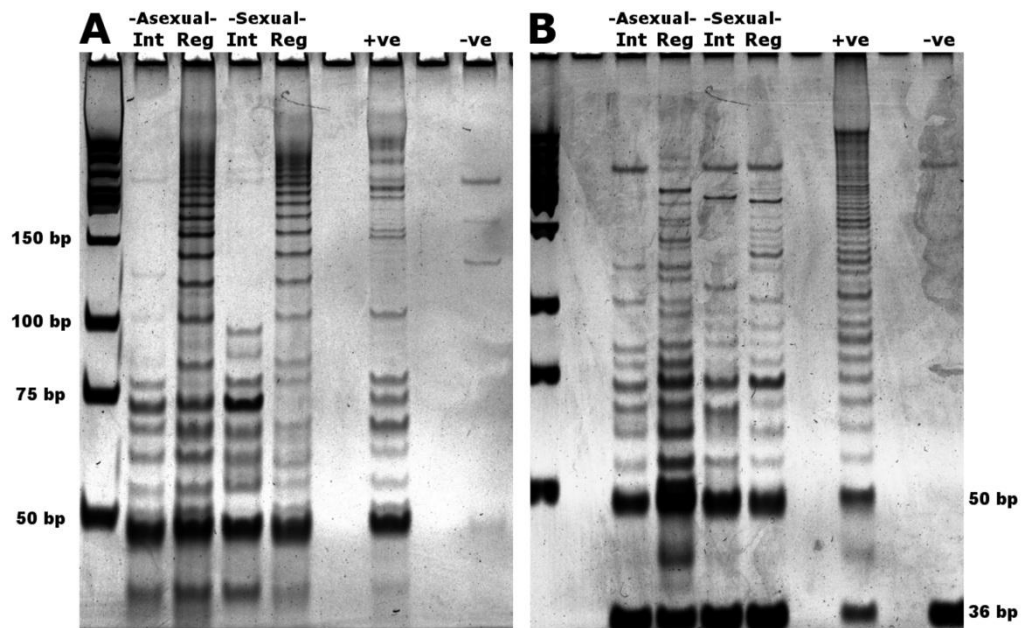


regenerating samples (Figure 3.2.5a and 3.2.5b), total proteins from regenerating worms produced longer TS products, as indicated by the size of the amplified bands. Inter-strain differences in intact samples were not noticeable. The regenerating sexuals had slightly fainter bands compared to the asexuals, this difference may be more apparent in Figure 3.2.5b where twice the amount of total protein was used in each sample. Appreciable difference in the size of the longest amplified products was not observed between strains.

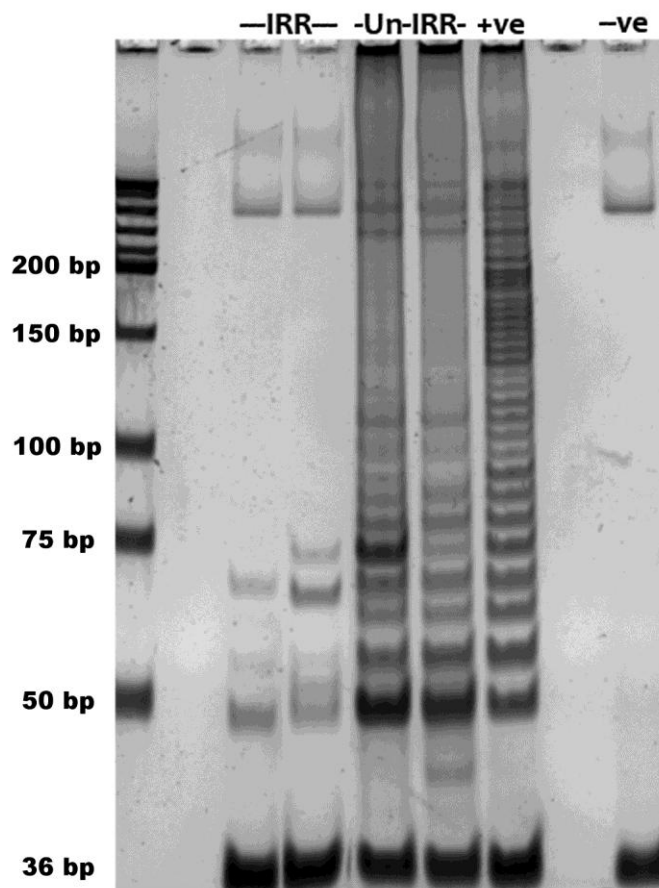
Total protein extracted from worms seven days after the treatment with 100Gy of gamma irradiation produced very little TRAP activity compared to the untreated controls.



**Figure 3.2.4 – Presence of TRAP assay inhibitor in the total protein extract.** PCR products for extended TS primers are fuzzy and do not increase by 6bp as seen in the HeLa cell extract positive control. Incubation with more than 0.05µg of the total protein reduced the presence of extended TS primers on the gel. These inhibitory effects can be removed by a phenol-chloroform extraction before the PCR step.



**Figure 3.2.5 – TRAP assay showing up-regulation of telomerase activity during regeneration.** Reactions were performed with **(A)** 0.5µg or **(B)** 1.0µg of total protein per sample (Samples in A and B were independently extracted). Int: Intact worm sample; Reg: Regenerating sample, 72hr after amputation. For each sample, total protein was extracted from 3 worms (~8mm each).



**Figure 3.2.6 – Telomerase activity after removal of neoblasts with 100Gy of gamma irradiation.** IRR: Irradiated samples; Un-IRR: Un-irradiated controls. Samples were extracted 7 days post treatment.

### 3.3 Discussion

The Bal-31 sensitivity of the (TTAGGG)<sub>n</sub> signals in the TRF analysis suggested that these signals were produced by the real terminal restriction fragments. The band produced by the internal *nanos* gene did not change in size after treatment. The disappearance of the signal in the 120 and 240 minute time points is likely due to the lower yield of ethanol precipitation as a result of decreased DNA content in the samples after the prolonged exonuclease degradation. The results of TRF analysis with 4-cutters have also ruled out the existence of internal (TTAGGG)<sub>n</sub> repeats larger than 2kb,

the lower end of the FIGE gel, since no signals of constant sizes were present between different samples. These results have validated the TRF technique as a method for studying telomere dynamics in *S. mediterranea*.

The 6-base-pair manner in which the TS substrate was elongated by the total protein extract is similar to that by the telomerase positive protein extract. In addition, the sensitivity of this elongation to RNase suggests the requirement for RNA components in the protein which processed the substrate. These results strongly suggest the presence of telomerase holoenzyme in *S. mediterranea* proteome.

The negative correlation between telomere lengths and age in sexual samples indicates that the homeostatic level of telomere maintenance is insufficient to prevent age-related telomere erosion. The fact that the new-born hatchlings of sexual worms have long telomeres while the parents which gave rise to these offspring have short telomeres suggests that telomere length is prevented from erosion in the germline, or the length resets during sexual reproduction, either in gametogenesis or embryogenesis. In contrast, rapid telomere attrition observed in regenerated sexual samples indicates insufficient telomere maintenance in somatic cells. Since mature germ cells apoptose during regeneration (Wang et al., 2007b), a majority of the TRF signals in regenerated samples should correspond to the somatic telomere lengths after rapid cell divisions. The difference between germline and somatic maintenance is similar to common multicellular telomere models such as human and mouse (LaBranche et al., 1998, Fiset and Chabot, 2001, Wagner et al., 2011). A different scenario to the common models is that the germ cells in planarians are thought to be differentiated progenies of the somatic stem

cells (Wang et al., 2007b). It is not certain whether there is a sub population of somatic stem cells with a high telomere maintenance level, which is pre-committed to the germ cell lineage, or if all somatic stem cells have the potency to form germ cells, and the telomere maintenance is only up-regulated after this commitment.

The telomere length reset occurs in asexual worms during regeneration, in contrast to the erosion of telomeres in the sexuals. The shorter telomeres observed in asexual samples with no recent regeneration events suggest insufficient telomere maintenance during homeostatic cell turnover, a characteristic shared between the strains. The periodic occurrence of regeneration events in asexual worms through reproduction by fission indicates that their telomeres are regularly replenished. The homogeneity of telomere length distribution after regeneration indicates that the maintenance event occurs in a majority, if not all, of body stem cells instead of a certain sub-population. Therefore the facultative sexual model discussed in section 3.1.3 is unlikely, since the maintenance event should occur in somatic cells instead of the *nanos* positive primordial germ cells. This somatic telomere maintenance circumvents the end-replication problem which limits the proliferation capacity of somatic cells, and therefore ensures the long-term survivability of the organism without passage through the germline. Evolution of this somatic maintenance is essential for adaptation to the agametic asexual life history.

The increase in TRAP activity during regeneration correlates with the boost of mitotic cell counts, which suggests a link between proliferation and telomerase regulation, possibly in the same cell cycle stage manner as seen in human and yeast, with the highest telomerase expression and

activities in S phase (Masutomi et al., 2003, Wellinger et al., 1993). The TRAP activity in intact samples displays the basal level of telomere maintenance during homeostatic turnover, this level is not sufficient to prevent telomere erosion, which is evident in the TRF analysis in both strains. The size of the telomerase-extended product increased in regenerating samples of both strains, indicating that both strains are capable of enhancing telomerase processivity during regeneration. The sexual regenerating sample had fainter bands compared to the asexual sample, suggesting lower telomerase activity in sexual regenerating worms. It is not clear whether the increase in TRAP activity in regenerating sexuals was due to the *nanos*-positive primordial germ cells which may have high maintenance but are low in number, or by all somatic stem cells which enhance telomerase to a less extent compared to the asexual stem cells. Although the significance of this inter-strain difference is yet to be assayed, the consequence of this difference is the fundamentally different telomere dynamics between the strains during regeneration.

In summary, the telomeric repeat unit in *S. mediterranea* has been confirmed to be (TTAGGG)<sub>n</sub>, the telomere length dynamics indicate a somatic telomere reset mechanism which is present in regenerating asexual worms but absent in the sexuals. Telomerase activity has been identified in this organism, and appears to be confined to radiation-sensitive stem cells. Both strains are capable of increasing telomerase processivity during regeneration; however the telomerase activity differs between the strains, causing telomere length reset in the asexuals and rapid telomere erosion in the sexuals.

## CHAPTER 4: DISCOVERY AND CHARACTERISATION OF *Smed\_TERT*, THE CATALYTIC SUBUNIT OF TELOMERASE IN *S.mediterranea*

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### 4.1 Introduction

#### **4.1.1 Discovery of *Smed\_Tert*, the reverse transcriptase subunit of telomerase**

Results from the previous chapter have provided evidence for the presence of telomerase activity in *S. mediterranea*. In order to study how cells control telomere maintenance and compare telomere biology between the strains, it is necessary to find the genes encoding the telomerase holoenzyme. Attempts to identify the homolog for the telomerase RNA subunit have failed because of the poor sequence similarity between species. However preliminary work by Daniel Felix has identified a short sequence similar to the catalytic reverse transcriptase subunit of telomerase (TERT, referred to as EST2 in *Saccharomyces cerevisiae*) of *Schizosaccharomyces pombe* in the planarian genome using TBlastN. Further bioinformatic analyses using gene prediction software GENSCAN and BLAST with multiple EST databases were performed, and the full length of the gene was identified and confirmed with reverse transcription-PCR and sequencing. The sequence of the protein was aligned with TERT in multiple organisms and most of the conserved sequence motifs were mapped, including those located in the reverse transcriptase domain and the telomerase specific region. Sequencing has also identified several alternatively spliced variants of the gene (details in Chapter 5).



#### **4.1.2 Expression patterns of *Smed\_Tert* in the sexual and asexual strains**

With the sequence of *Smed\_Tert* identified, in-situ hybridisation was performed in intact, regenerating, and irradiated samples of both strains with a 5'-DIG-labelled RNA probe targeting at a consensus sequence of all known *Smed\_Tert* isoforms. As a follow-up from the TRAP experiment in irradiated animals in Chapter 3, qRT-PCR on the transcription level of *Smed\_Tert* in irradiated samples was also performed to test for consistency with the previous protein assay.

#### **4.1.3 Inhibition of *Smed\_Tert* expression by RNA interference**

Double stranded RNA was artificially synthesised from a 601bp consensus region between all *Smed\_Tert* transcripts, this spans the Motif C, D, and E of the reverse transcriptase domain. The dsRNA was injected into the gut branches of the worms from an area anterior to the pharynx, and the effect of the RNAi was confirmed by a TRAP assay on samples collected after 15 weeks of treatment. Control samples were injected with the same amount of dsRed dsRNA. The asexual strain was used in this experiment due to the consistent telomere lengths between individuals. TRF analyses were performed in samples collected as the RNAi progressed. Some samples were withdrawn from the RNAi after 18 weeks of injections and their telomere lengths were measured after 12, 21, and 33 weeks of telomerase restoration. All TRF analyses were done on regenerating samples for maximum difference between erosion and reset. The relative telomere lengths between the RNAi samples, the controls, and the telomerase-restored samples were confirmed by qPCR with the same technique described in Section 3.1.3.

#### 4.1.4 Proliferation assays on *Smed\_Tert* RNAi samples

The TRAP results from the previous chapter have shown a strong link between telomerase activity, proliferation and proliferative cells. In order to understand the effect of the *Smed\_Tert* inhibition on proliferation, whole mount immunohistochemistry was conducted with an antibody against phosphorylated serine 10 in histone H3, which was previously shown to specifically mark cells in M phase (Gurley et al., 1978, Paulson and Taylor, 1982, Hendzel et al., 1997, Newmark and Sánchez Alvarado, 2000). It is necessary to separate the effect of the absence of Smed\_TERT protein with the effect of short telomere on proliferation, and therefore the immunostaining was done on samples with two different durations of RNAi treatment, 15 and 46 weeks. Samples treated with 15 weeks of RNAi had little residual Smed\_TERT activity while their telomeres were on average 10kb longer than those in the 46 weeks samples (shown in Section 4.2.3). The number of cells stained positive with the marker was compared to the number in the controls injected with dsRed dsRNA for the same duration. Counts in intact samples indicate the basal level of cell division, while counts in regenerating samples show the ability of the worms to increase proliferation in response to injuries.

## 4.2 Results

Whole mount in situ hybridisations were conducted with help from Farah Jaber Hijazi, while the phosphorylated histone H3 ser10 immunohistochemistry was performed in collaboration with Chen Chen.

#### 4.2.1 Discovery of *Smed\_Tert* gene in *S. mediterranea* genome

TBlastN and multi-species alignment using human, mouse, and fission yeast TERT protein sequences produced a 216bp homologous sequence hit on the negative strand of Contig v31.000666 (42404 – 42189bp) of the genome assembly (see Figure 4.2.1). This sequence encodes a part of the canonical reverse transcriptase domain of Smed\_TERT, which locates at the C-terminal half of the protein in all studied organisms. Genscan software had predicted a 1362aa protein located between 59776 – 5711bp of the contig, and the nearest ESTs mapped to the predicted transcript on each side of the TBlastN hit were used as primer targets for PCR amplification from cDNA. The 668bp long EST PL08003A2F11 (Zayas et al., 2005) locates 3' to the TBlastN hit on the predicted transcript, and contains a 75bp overlap with the hit. While the 722bp long ec1.13248.001 (Robb et al., 2008) was mapped 5' to the hit. A forward primer (TERT\_F7) was designed from ec1.13248.001 and a reverse (TERT\_R) from PL08003A2F11, which produced multiple PCR amplicons from cDNA synthesised from both the sexual and asexual strains (details of different amplicons are included in Chapter 5 and Figure 5.2.1). Sequencing of the longest cloned amplicon revealed an 1833bp long partial transcript which shows high homology to TERT protein in BlastX (organism of the highest hit: *Strongylocentrotus purpuratus*,  $e = 3 \times 10^{-14}$ ). When the unamplified regions in both ESTs were included into the transcript, an in-frame STOP site was identified close to the 3' end of PL08003A2F11, with 24bp of 3' UTR. The START site was identified as the closest ATG site in frame 3' to a STOP site at the 5' end of ec1.13248.001, with 23bp of 5' UTR. The lengths of UTRs were later confirmed by 5' and 3' RACE.

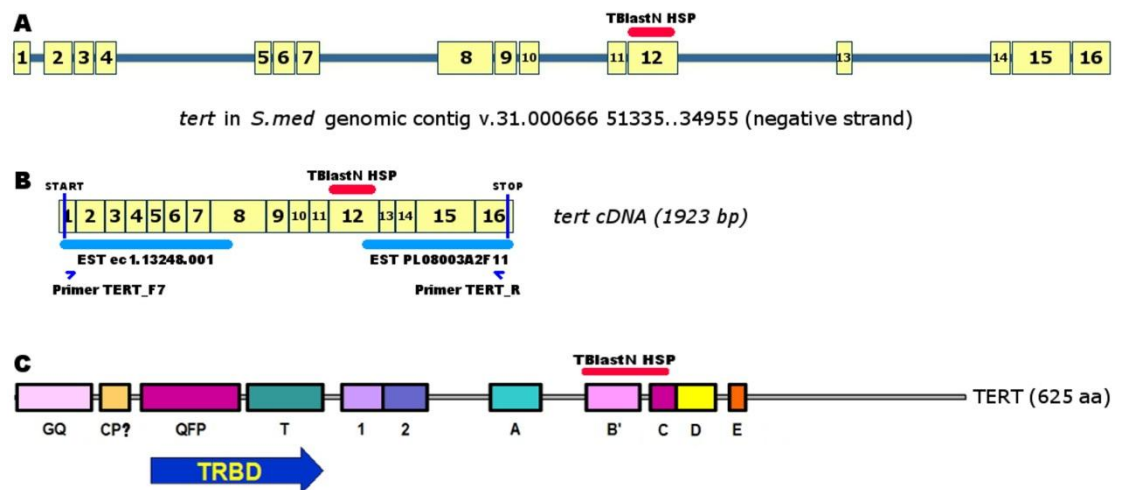
In summary, *Smed\_Tert* is located at 51308 – 34955bp of Contig v31.000666 in the *S. mediterranea* genome, with the full-length transcript 1895bp in length, which is translated into a 625aa protein. The locations of exons and introns are listed in Table 4.2.1.

| Exon/Intro<br>n | Location on<br>v31.000666 | Size<br>(bp<br>) | Exon/Intro<br>n | Location on<br>v31.000666 | Size<br>(bp<br>) |
|-----------------|---------------------------|------------------|-----------------|---------------------------|------------------|
| Exon 1          | 51269..51308              | 40               | Exon 9          | 44172..44268              | 97               |
| Intron 1        | 50958..51268              | 311              | Intron 9        | 44126..44171              | 46               |
| Exon 2          | 50835..50957              | 123              | Exon 10         | 44037..44125              | 89               |
| Intron 2        | 50787..50834              | 48               | Intron 10       | 42530..44036              | 1507             |
| Exon 3          | 50702..50786              | 85               | Exon 11         | 42450..42529              | 80               |
| Intron 3        | 50660..50701              | 42               | Intron 11       | 42405..42449              | 45               |
| Exon 4          | 50570..50659              | 90               | Exon 12         | 42187..42404              | 218              |
| Intron 4        | 47497..50569              | 3073             | Intron 12       | 38675..42186              | 3512             |
| Exon 5          | 47428..47496              | 69               | Exon 13         | 38612..38674              | 63               |
| Intron 5        | 47389..4742               | 39               | Intron 13       | 35569..3861               | 304              |

|          |             |     |  |           |             |
|----------|-------------|-----|--|-----------|-------------|
|          | 7           |     |  | 1         | 3           |
| Exon 6   | 47293..4738 | 96  |  | Exon 14   | 35483..3556 |
|          | 8           |     |  | 8         | 86          |
| Intron 6 | 47250..4729 | 43  |  | Intron 14 | 35438..3548 |
|          | 2           |     |  | 2         | 45          |
| Exon 7   | 47153..4724 | 97  |  | Exon 15   | 35184..3543 |
|          | 9           |     |  | 7         | 254         |
| Intron 7 | 44555..4715 | 259 |  | Intron 15 | 35123..3518 |
|          | 2           | 8   |  | 3         | 61          |
| Exon 8   | 44315..4455 | 240 |  | Exon 16   | 34955..3512 |
|          | 4           |     |  | 2         | 168         |
| Intron 8 | 44269..4431 | 46  |  |           |             |
|          | 4           |     |  |           |             |

**Table 4.2.1 – Locations of exons and introns of *Smed\_Tert* gene on Contig v31.000666.**

Multi-species alignment of TERT had identified most of the conserved sequence motifs of known functions (Figure 4.2.2 and Appendix A), including the canonical reverse transcriptase region (1, 2, A, B', C, D, and E) and the telomerase-specific signatures (GQ, CP, QFP, and T). The RNA binding domain was identified by BlastP. High homology was found from Motif QFP to E, however the N-terminal part of the protein contains sequences highly diverged from other species and both Motif GQ and CP were hardly aligned. Motif DAT could not be identified by this alignment. Unlike Trt-1 in *C. elegans*, the full length Smed\_TERT contains a well aligned QFP motif.

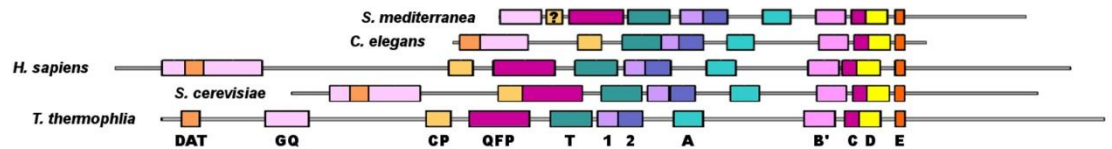


**Figure 4.2.1 – Gene and protein annotations of Smed\_TERT. (A)**

Location of *Smed\_Tert* in the *S. mediterranea* genome assembly version 3.1, introns were shrunk to 1/5 of their actual sizes for the sake of this illustration. The “TblastN HSP” marks the region of the highest homology between multiple species (for alignment see Figure 4.2.2 and Appendix A).

**(B)** The 1923bp full length transcript of *Smed\_Tert*, with the two closest ESTs mapped on either side of the TblastN hit site. **(C)** Protein structure of

*Smed\_TERT*. The multi-species conserved motifs include the canonical reverse transcriptase region (1, 2, A, B', C, D, and E) and the telomerase-specific signatures (GQ, CP, QFP, and T). Motif CP cannot be precisely mapped due to low sequence similarity. TRBD: Telomerase RNA Binding Domain.



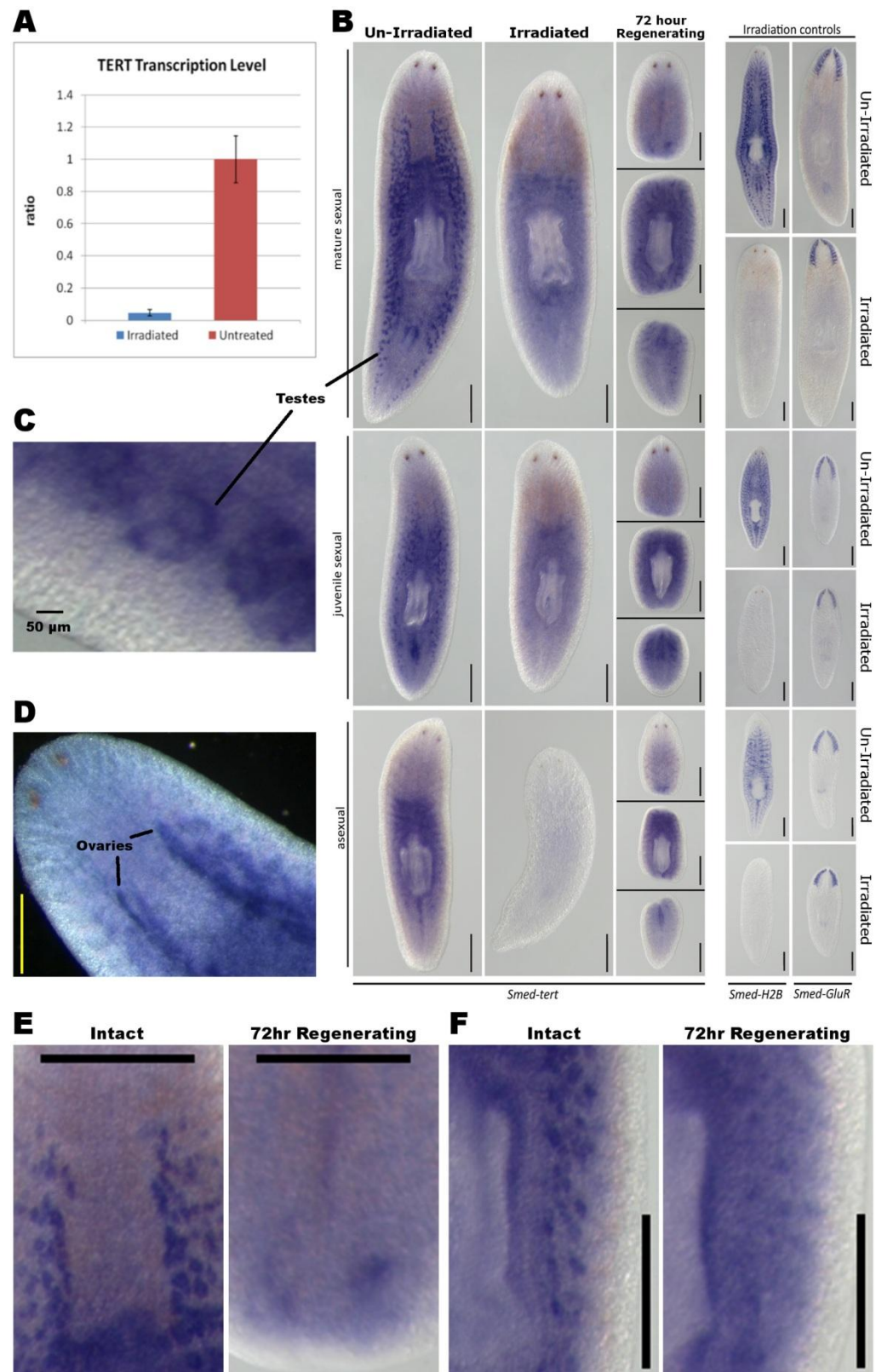
**Figure 4.2.2 – Multi-species comparison of TERT protein structure.**

Sequence 5' of the GFP motif in *S. mediterranea* has low homology to TERT in other species and the motifs could not be precisely mapped (for details of the sequence alignment see Appendix A).

#### 4.2.2 Expression pattern of *Smed\_Tert*

qPCR on the total transcription level of all *Smed\_Tert* isoforms (using TERT\_qPCR\_F3/R3 primer set) showed an average of 95.31% decrease (t-test, two tailed, unequal variance,  $p=0.00701$ ) 7 days after treatment with 100Gy of gamma irradiation, comparing to the mock-irradiated controls (see Figure 4.2.3a). The experiment was done in three replicates on batches of five worms.

Whole mount in-situ hybridisation of *Smed\_Tert* showed expression in the testes, ovaries, and gonadopore of mature sexual worms ( $n=7$ ) (see Figure 4.2.3b-d). This pattern is sensitive to irradiation, and regressed in samples fixed at 72 hours post amputation. Expression could also be observed in primordial reproductive cells in juvenile sexual worms ( $n=7$ ), which also disappeared when irradiated and during regeneration. In asexual samples a trans-parenchyma pattern, concentrated in an area surrounding pharynx and the posterior mid-line, was observed ( $n=7$ ). The expression could also be eliminated by irradiation. In 72 hour post-amputation regenerating samples the pattern did not differ from intact samples. The trans-parenchyma pattern may also exist in the sexuals, however this could not be distinguished from the strong background in these samples.



**Figure 4.2.3 – Expression pattern of *Smed\_Tert* in both strains. (A)** Transcription level the *Smed\_Tert* in asexual worms 7 days after irradiation. **(B)** Whole mount in-situ hybridisation of *Smed\_Tert* in mature

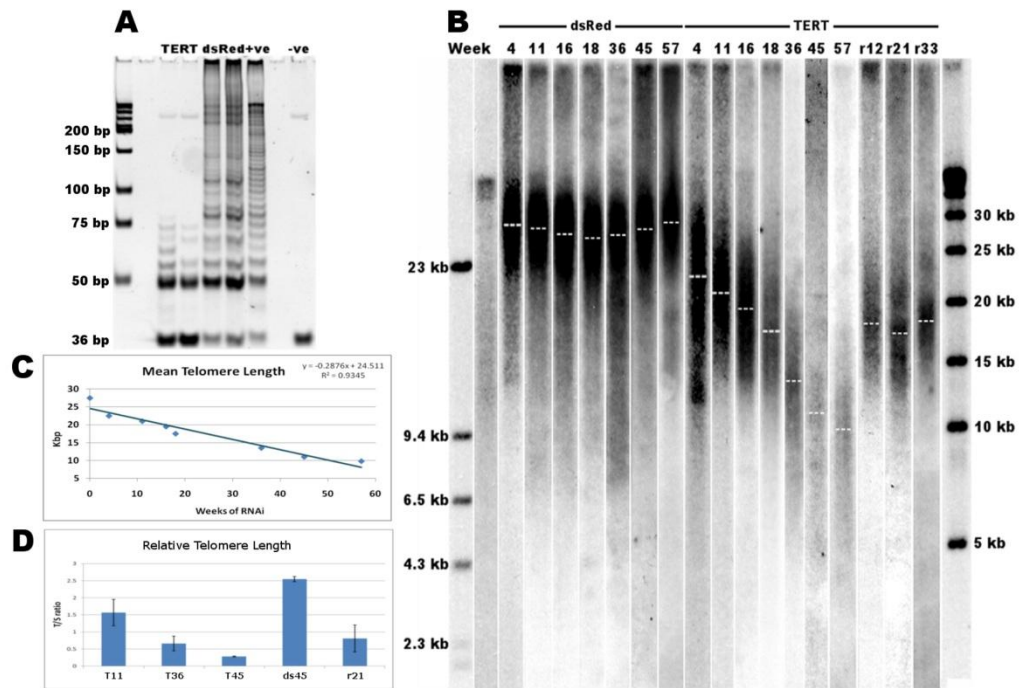


sexual, juvenile sexual and asexual worms. All irradiated samples were treated with 85Gy of gamma-irradiation, and fixed on the 7<sup>th</sup> day post-treatment. Irradiation control marker *Smed-H2B* represents a cell population sensitive to irradiation, while *Smed-GluR* represents a population which is insensitive. **(C)** High magnification image of *Smed\_Tert* expression in the periphery of mature testes. **(D)** Ventral view of a mature sexual sample showing *Smed\_Tert* expression in the ovaries. **(E – F)** Magnified view of *Smed\_Tert* expression in homeostatic and regenerating sexual mature samples, showing diminishing expression of the gene in germ cells as they apoptose. **(E)** Anterior region between the photoreceptors and pharynx. **(F)** Trunk region close to the pharynx. Scale bar: 0.5mm.

#### 4.2.3 Inhibition of *Smed\_Tert* expression by RNAi

TRAP assay on regenerating asexual samples extracted after 15 weeks of injections with *Smed\_Tert* dsRNA showed observable decrease in telomerase activity (see Figure 4.2.4a). This is accompanied by progressive reductions in telomere lengths, as well as diffusion in the distribution of lengths, as shown in Figure 4.2.4b. The calculated mean telomere lengths, indicated by the white dotted lines, in samples collected as the RNAi treatment prolongs, were analysed in Figure 4.2.4c. The reduction showed a good correlation with the duration of RNAi ( $R^2 = 0.9345$ ), and occurred at a rate of approximately 290bp per week. After removing the RNAi, the telomeres remained at the mean length when the withdrawal occurred (week 18). As the recovery progresses the length distribution was restored to the homogeneity seen in the dsRed controls, however the mean lengths remained unchanged. Three individual samples were measured at each time point.

Telomere lengths measured by qPCR also showed relative decreases in *Smed\_Tert* RNAi treated worms (see Figure 4.2.4d). With the same reference DNA as used in Chapter 3, samples treated with 11 weeks of RNAi had a mean T/S value of 1.57 (s.d. of mean = 0.388), while the 36 and 45 weeks samples had 0.66 and 0.28 (s.d.=0.216 and 0.0161), respectively. The control samples, treated with 45 weeks of dsRed RNAi, had a mean T/S of 2.55 (s.d.=0.0750). Samples recovered after 21 weeks of RNAi withdrawal from week 18 had a mean T/S of 0.81 (s.d.=0.392). Three individual samples were measured at each time point.

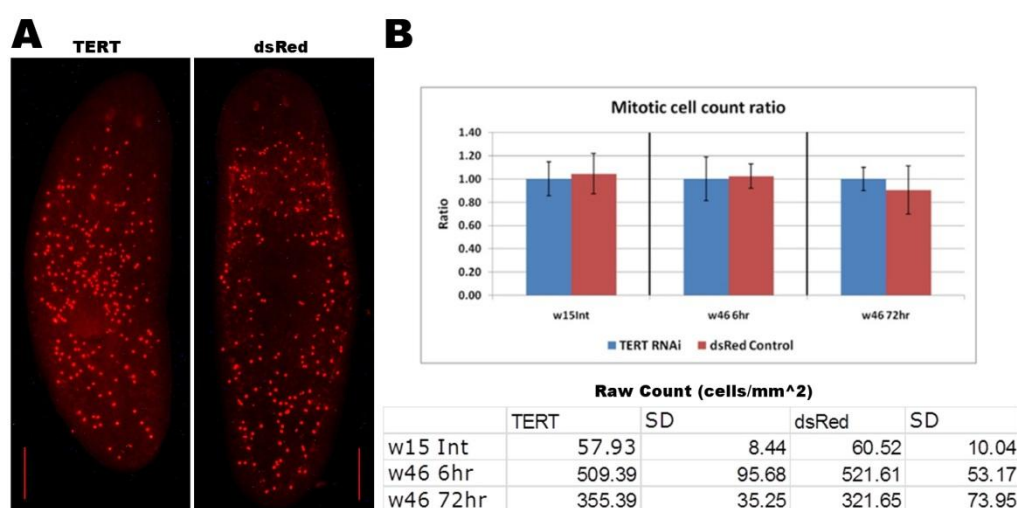


**Figure 4.2.4 – Effect of *Smed\_Tert* RNA interference on the telomerase activity and telomere length. (A)** TRAP activity in 72 hour post-amputation regenerating samples after 15 weeks of RNAi. Two independent protein extractions were performed for each RNAi-treated group, with 3 worms in each extraction. **(B)** Progressive shortening of telomere in samples injected with *Smed\_Tert* dsRNA. After removing RNAi (Lane r12, r21 and r33, which correspond to the number of week post removal) the telomere is stabilised to the length at the time of withdrawal (week 18). White dotted lines represent the mean telomere length of each sample, defined as  $\Sigma(OD_i)/\Sigma(OD_i/L_i)$  as previously described (Harley et al., 1990). Each lane is a representative of a triplicate experiment using three individual worms. **(C)** Mean length of telomere versus the length of RNAi treatment. An average erosion rate of approximately 290bp per week is observed. **(D)** qPCR analysis for the relative telomere lengths in worms treated with *Smed\_Tert* RNAi (T11, T36 and T45) or dsRed control (ds45). r21 refers to samples taken after 21 weeks of recovery from RNAi since the

withdrawal in week 18. Three individual samples were measured at each time point.

#### 4.2.4 Effect of *Smed\_Tert* RNAi on cell proliferation

The phosphorylated histone H3 serine 10 immunostaining had found no significant difference in mitotic cell counts between the *Smed\_Tert* RNAi treated samples and the dsRed controls in all sample groups including intact worms on Week 15 and regenerating worms reaching their two mitotic peaks on Week 46 (n=10, p=0.562, 0.371 and 0.148, respectively; t-test, two tailed, unequal variance) (see Figure 4.2.5).



**Figure 4.2.5 – Mitotic cells counted from the phosphorylated histone H3 ser10 immunohistochemistry. (A)** Examples of the immunofluorescence signal in Week 15 intact samples. **(B)** Cell counts shown as ratios to the counts in the *Smed\_Tert* RNAi samples in each individual sample group. Raw cell counts are shown below. Ten individual samples were included in each time point. Int: intact; 6hr and 72hr: regenerants reaching their first and second mitotic peak, respectively.

## **4.3 Discussion**

### **4.3.1 *Smed\_Tert* gene, transcript, and protein**

The 1895bp transcript of *Smed\_Tert* is encoded by its gene which spans 16.354kb in the genome. The presence of enormously sized introns and some small exons (for example, the 61bp Exon 13 is flanked by a 3512bp 5' intron and a 3043bp 3' intron) have been an obstacle to the mapping of ESTs to the genome. Fortunately the Genscan software was able to produce a predicted transcript to which the two ESTs could be mapped, and the beginning and the end of the ORF happened to be contained in each EST. The two ESTs originate from separate databases, indicating the discrepancies between different sequencings and assemblies of the *S. mediterranea* transcriptome.

Sequencing of cloned amplicons from TERT\_F7 and TERT\_R have revealed various alternatively spliced isoforms of *Smed\_Tert*, which will be shown and analysed in detail in Chapter 5. Some isoforms appeared to lack Exon 4, 5, and 6, which encodes for the QFP and T motifs of the telomerase RNA-binding domain (TRBD). This domain is conserved across eukaryotic kingdoms (Xia et al., 2000), however it is shortened in *C. elegans* due to the absence of the QFP motif (Meier et al., 2006). *S. mediterranea* has naturally occurring isoforms of *Smed\_Tert* with full-length or truncated TRBDs, indicating a possible branching point of TERT evolution in invertebrates with short TRBD. The QFP motif has been previously shown to interact with the telomerase RNA subunit (Friedman and Cech, 1999), together with Motif CP and T, and is essential for telomerase assembly (Xia et al., 2000, Lai et al., 2001, Bosoy et al., 2003). However the lack of QFP domain in *C. elegans* does not impair the function of its telomerase. The

functions of the naturally occurring *Smed\_Tert* isoforms in *S. mediterranea* are yet to be studied. We hypothesise that the isoforms with short TRBD possess different target specificity, or may act as a competitive inhibitor of the full-length isoform for telomere binding.

#### 4.3.2 Cell specificity of *Smed\_Tert* expression

Consistent with the elimination of TRAP activity after irradiation shown in Chapter 3, transcription of *Smed\_Tert* three days after lethal irradiation decreased to less than 5% of that in mock-irradiated controls, providing further evidence for the exclusive expression of *Smed\_Tert* in proliferative cells. Whole mount in-situ hybridisation showed high expressions in testes, ovaries, and gonadopore in the sexual strain. High magnification image suggests specific expression in the periphery of the testes, resembling the pattern of a previously reported gene *hnRNPA2*, which is enriched in spermatogonial cells and early spermatocytes (Wang et al., 2010). Regression of *Smed\_Tert*-expressing testes in 72hr regenerating sexual worms resembles the regression pattern of *T-plastin*, which is expressed specifically in the spermatocytes and spermatids (Zayas et al., 2005, Wang et al., 2007b). In contrast with *nanos* which labels all cells committed to the germline property, including precursor cells, and the expression pattern does not regress to the same extent as *T-plastin* during regeneration (Sato et al., 2006, Wang et al., 2007b, Handberg-Thorsager and Salo, 2007). It seems likely that *Smed\_Tert* is up-regulated only in proliferating gamete-producing germ cells in mature sexual worms, and these cells undergo programmed cell death during regeneration (González-Estévez and Saló, 2010). In sexual juveniles the expression is high in the primordial germ cells, which is similar to the pattern of *nanos* positive germline cells. In the 72hr regenerating juvenile samples these cells lose

*Smed\_Tert* expression. It is therefore likely that the germline primordial cells are high in telomerase when they proliferate during development of reproductive organs, and become relatively quiescent and low in telomerase during regeneration. Although previous studies have not shown the proliferation pattern of *nanos* positive cells during different stages of regeneration, it can be hypothesised that proliferation of these cells is restored after 7 days post amputation, when the *T-plastin* positive cells begin to reappear in mature worms (Wang et al., 2007b). In summary, it is proposed that the telomerase in sexual planarians is highly expressed only in rapidly proliferating cells with germline properties, and these cells are either removed (*T-plastin*<sup>+</sup>) or do not divide (*nanos*<sup>+</sup>) during regeneration. Double in-situ hybridisations with *Smed\_Tert* and *T-plastin*, as well as *Smed\_Tert* and *nanos* in various time points of regenerating sexual planarians, together with the locations of phospho-histone H3 Ser10 positive cells in these samples, are required to test this theory.

With strong background hybridisation it is not currently possible to observe the expression of *Smed\_Tert* in somatic stem cells in sexual samples. Future TRAP and qPCR analysis with sexual worms treated with *nanos* RNAi, as well as optimisation of *Smed\_Tert* in-situ hybridisation, are required for further understanding of the difference in the telomerase activity between the germline and somatic stem cells.

In the asexual strain high expression of *Smed\_Tert* was found in somatic stem cells concentrated around the pharynx and the posterior mid-line. In contrast with the pattern in sexual worms, expression was not found in *nanos* positive presumptive germ cells in the asexuals and appeared to be present ubiquitously in somatic stem cells. This supports the observation

from the TRF analysis, which suggests that telomeres of all stem cells are replenished, as opposed to maintenance in a confined sub-population. Current in-situ results in regenerating samples did not show obvious *Smed\_Tert* up-regulation, however the transcription level of the gene will be studied in more precision with qPCR in Chapter 5.

#### 4.3.3 Inhibition and restoration of *Smed\_Tert* transcription

Inhibition of *Smed\_Tert* transcription by dsRNA injection has reduced the level of telomerase activity sufficiently to cause rapid erosion of telomeres and lack of telomere reset during regeneration. However the RNAi treated animals did not develop the *Tert* knockout phenotypes observed in *Xenopus laevis* and mouse, which include impaired anterior-posterior axis formation and homeotic transformation (Park et al., 2009). No physical phenotypes or defects in regeneration were observed in the knocked-down samples, possibly due to the presence of residual Smed\_TERT protein which is inevitable with the RNAi technique. In the Week 57 samples with a mean telomere length of less than 10kb, the worms grew and regenerated normally, suggesting that the mortality stage has not yet been reached.

An unanticipated result was observed in samples recovering from *Smed\_Tert* RNAi, where the mean telomere lengths were reset to a new size determined by the length of telomeres at the time of withdrawal. Restoration of telomerase activity did not increase the mean telomere length, but only restores the homogeneity of the length distribution after regeneration. This result raises a question about how a cell decides the target telomere length to which its telomerase needs to reach during regeneration, and how the lower part of TRF length distribution in pre-regeneration samples disappears after regeneration. One may speculate



that the level of telomere maintenance between individual cells differs depending on their relative telomere lengths. Assuming a certain sub-population of stem cells always has the longest telomere in the body, and other types of stem cells have a way to detect the relative length of their telomeres to the longest, and enhance telomere maintenance accordingly. This enhancement may involve increase in transcription of full-length *Smed\_Tert*, reduction in telomerase inhibitors, improved telomerase assembly in the Cajal body, better recruitment of telomerase in late S phase, and/or post-translational regulations of telomerase (see Chapter 1 for reviews of these mechanisms).

Another possible explanation for the length distribution dynamics during regeneration is that the enhanced telomerase activity is enough to prevent telomere erosion in the cell population with longest telomeres. Both stem cells and end differentiated cells with short telomeres may be subjected to programmed cell death, as suggested by the TUNEL assay in regenerating planarians in previous studies (Hwang et al., 2004, Pellettieri et al., 2010), and the body is replaced by new progeny of the long-telomere stem cells. A first burst of cell death occurs at 1-4 hours post amputation, followed by the first mitotic peak at 4-6 hours; the second peak of both cell deaths and cell divisions occur at approximately 3 days post-amputation. These coinciding events support the replacement theory of the telomere reset. The fact that cells with restored telomerase activity after RNAi removal remained with stable short telomeres is also in favour of the hypothesis that telomerase prevents erosion instead of elongates telomeres in the cell population with long telomeres. Although no TUNEL patterns in regenerating sexual planarians have been published to date, the cellular events during regeneration in sexual worms should be similar to those in

the asexuals. It is therefore difficult to explain the increased heterogeneity in telomere length distribution after regeneration in the sexuals with the replacement theory, since the TRF result suggests against the predominance of fresh long-telomere progeny cells in regenerated sexual worms.

Experiments can be designed to test the enhanced-maintenance theory and the replacement theory (not necessarily mutually exclusive). Optimisation of *Smed\_Tert* in-situ hybridisation or generation of stable transgenic lines with reporter-tagged Smed\_TERT is required for localisation and quantitative studies. Double in-situ with *Smed\_Tert* and telomeric PNA probes should give information about the level of telomere maintenance in cells with different telomere lengths, while double staining with TUNEL and telomeric PNA should reveal whether cells with short telomeres are major subjects of apoptosis during regeneration.

#### **4.3.4 Smed\_TERT and cell cycle progression**

The data in Figure 4.2.5 indicates that knock-down of *Smed\_Tert* transcription, and the resulting insufficiency of Smed\_TERT protein, do not affect the basal proliferation counts in planarians; and at a mean telomere length of approximately 10kb, the cells can enter proliferation normally without arrest at cell cycle checkpoints. These results are consistent with the lack of observable phenotypes and unaffected regeneration capacities in RNAi samples (experiment concluded in Week 60).

## CHAPTER 5: NATURALLY OCCURRING VARIATIONS OF Smed\_TERT

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### 5.1 Introduction

#### 5.1.1 Alternative splicing isoforms of *Smed\_Tert* in other organisms

Transcription variants of *Tert* have been previously identified in human (Kilian et al., 1997), with alternative splice sites located solely in the reverse transcriptase region of the gene. One of the variants (*hTerta*) utilises an alternative splice acceptor site in Exon 6, resulting in an in-frame deletion of a part of the conserved Motif A, and possesses a dominant negative function when ectopically expressed in telomerase-positive human cells (Colgin et al., 2000, Yi et al., 2000). Similar inhibitory properties were also found in experimentally mutated human TERT proteins with single amino acid substitutions such as D712A (Motif A) and D868/9A (Motif C) (Zhang et al., 1999). In addition to the  $\alpha$  variant, *hTert $\beta$*  lacks Exon 7 and 8 which locate at the linker region between Motif A and B' (Kilian et al., 1997). Exclusion of this region results in a premature STOP codon in Exon 10, which truncates the protein. Both *hTert $\beta$*  and *hTerta $\beta$*  (which lacks both regions) have no catalytic activities and do not act as dominant negative inhibitors (Yi et al., 2000). The other isoforms currently identified contain intronic insertions, for which no detailed functional studies have been published.

A recent study in a chicken MDV lymphoma-derived MSB-1 T-cell line has identified 27 alternatively spliced variants of *chTert* (Amor et al., 2010).

Five of those variants are in-frame exon-exclusion transcripts, which were shown to be non-functional by TRAP assay when reconstituted *in vitro*. The remaining 22 variants possess premature STOP codons, 19 of which have the codon more than 50bp upstream of the 3' splice junction and were therefore predicted as targets for nonsense-mediated decay (NMD). The most abundant prematurely stopped variant was confirmed as a NMD target by RNAi against the NMD pathway. The low telomerase activity in normal T-cells was shown to be a result of predominant expression of in-frame variants. Increase in the telomerase activity during induced lymphomagenesis coincides with a proportional increase in the level of constitutively spliced *chTERT* over other variants, together with an increase in the prematurely stopped inactive variants over the in-frame inactive variants.

#### 5.1.2 *Smed\_Tert* isoforms in *S. mediterranea*

Cloning and sequencing of RT-PCR products from cDNA synthesised from both strains have so far identified two alternatively spliced sites in *Smed\_Tert*. The 255bp Alternative Region I contains Exon 4, 5, and 6 of the constitutively spliced transcript, which encode for the conserved Motif QFP and a part of Motif T of the TRBD domain. While the 298bp Alternative Region II is in the RT domain and consists of Exon 11 and 12, which covers the linker region between Motif A and B', as well as Motif B', C, and a part of Motif D (see Figure 5.2.1c). Isoforms lacking Alt Region I appeared to be in-frame, whereas those lacking Alt Region II possess a premature STOP codon due to frame shift.

In order to test whether transcriptional control plays a role in the up-regulation of telomerase and telomere length lengthening during

regeneration observed in Chapter 1, qPCR primers were designed to specifically amplify isoforms which contain (+ve primers), or do not contain (-ve primers), the alternative regions. A +ve primer set has at least one of the oligonucleotide located within the alternative site, while a -ve primer set has one of the oligonucleotide sitting across the splicing boundary at its 3' end. qPCR reactions were performed in intact and regenerating samples of asexual, juvenile sexual, and mature sexual strains.

For the total transcription level of all *Smed\_Tert* isoforms, a primer set TERT\_qPCR\_F3/R3 was designed to amplify a region found in all isoforms. The region locates at the 3' end of the transcripts and therefore the 3' bias nature of OligodT-based cDNA has a minimised effect, and the copy number of the amplicon corresponds to the real total level of *Smed\_Tert* transcription in the sample.

## **5.2 Results**

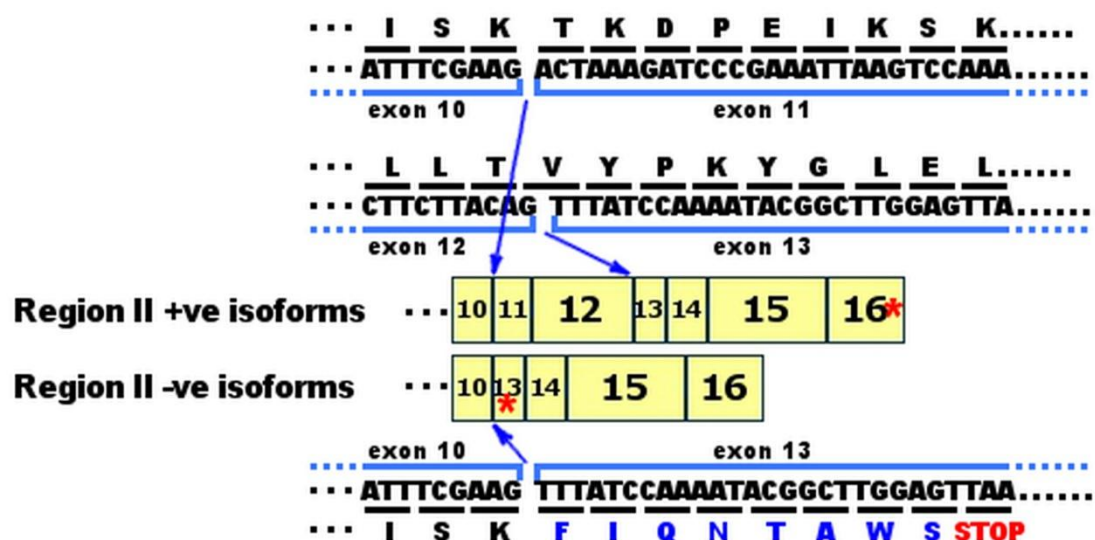
### **5.2.1 Currently identified alternatively spliced isoforms of *Smed\_Tert***

RT-PCR using TERT\_F7 and TERT\_R produced multiple products with various sizes in all samples (see Figure 5.2.1b). A region between 1kb to 2kb on the gel was cut and extracted, and the products from one individual worm of each strain were cloned separately into pCR2.1 TOPO vectors (Invitrogen). Sequencing of the randomly picked positive colonies revealed the identity of different sized products as transcription variants of *Smed\_Tert* given rise by alternative splicing. Isoform 1 has a PCR product size of 1833bp, it is the longest isoform and therefore is thought to be the constitutively spliced full-length transcript. Isoform 2 lacks the 255bp

Alternative Region I and its PCR product size is 1578bp. Isoform 3 lacks the 298bp Alternative Region II and the product is 1535bp in size. Isoform 4 contains neither of the alternative regions and its product size is 1280bp. The ORF of each isoform was identified bioinformatically. Isoform 1 and 2 are both in-frame and can be translated into 625aa and 540aa proteins, respectively. Both Isoform 3 and 4 contain a premature STOP codon in Exon 13 due to the frame-shift caused by the exclusion of Alternative Region II (see Figure 5.2.2), their ORF size are therefore drastically reduced and the proteins they encode are 353aa and 268aa, respectively.



**Figure 5.2.1 – Regions of alternative splicing in *Smed\_Tert* and currently identified *Smed\_Tert* isoforms. (A)** Alternative Region I includes exon 4, 5 and 6, while Alternative Region II includes exon 11 and 12. **(B)** RT-PCR products from both strains using the TERT\_F7 and TERT\_R primer set. The gel region between 1.25kb and 2kb was cloned and 4 isoforms, lacking either or both Alt regions, were identified by sequencing. Isoform 3 and 4 have an alternative STOP site due to frame shift (see Figure 5.2.2). **(C)** Alt Region I locates at the QFP motif and a part of the T motif of the Telomerase RNA subunit Binding Domain (TRBD), Alt Region II is an essential part of the reverse transcriptase domain.



**Figure 5.2.2 – Alternative STOP site in Region II -ve *Smed\_Tert* isoforms.** Isoform 3 and 4 have a premature STOP site (indicated by an asterisk) in exon 13 due to a frame shift caused by the exclusion of exon 11 and 12 which truncates the reverse transcriptase domain of *Smed\_TERT* (see Figure 5.2.1c).

### 5.2.2 Single nucleotide variations between haplotypes

Substitutions at the single nucleotide level were observed between different RT-PCR clones derived from an individual asexual worm. These mutations did not appear to be isoform-specific. The currently identified SNPs are listed in Table 5.2.1.

| Position<br>(bp)       | Strain  | Nucleotide | AA.<br>Residue | Abundance | Haplotype |
|------------------------|---------|------------|----------------|-----------|-----------|
| 179-180<br>(Motif CP)  | Sexual  | GA         | I63            | 15/15     | -         |
|                        | Asexual | GG         | V63            | 5/15      | M1 & M2   |
|                        | Asexual | CA         | I63            | 10/15     | S         |
| 605<br>(T-1<br>linker) | Sexual  | C          | I205           | 15/15     | -         |
|                        | Asexual | T          | I205           | 15/15     | M1/2 & S  |



|                       |         |   |           |       |         |
|-----------------------|---------|---|-----------|-------|---------|
| 635<br>(Motif 1)      | Sexual  | T | S215      | 15/15 | -       |
|                       | Asexual | C | S215      | 5/15  | M1 & M2 |
|                       | Asexual | T | S215      | 10/15 | S       |
| 837<br>(2-A linker)   | Sexual  | C | L282      | 15/15 | -       |
|                       | Asexual | G | V282      | 5/15  | M1 & M2 |
|                       | Asexual | C | L282      | 10/15 | S       |
| 1312<br>(Motif D)     | Sexual  | A | K440      | 12/15 | -       |
|                       | Asexual | T | I440      | 3/15  | M1      |
|                       | Asexual | A | K440      | 11/15 | M2 & S  |
| 1712<br>(3' terminal) | Sexual  | T | S574 / NA | 15/15 | -       |
|                       | Asexual | G | S574 / NA | 3/15  | M1      |
|                       | Asexual | T | S574 / NA | 12/15 | M2 & S  |

**Table 5.2.1 – Asexual strain-specific single nucleotide variations of *Smed\_Tert*.** Since the mutations are present only in certain RT-PCR clones, these clones are named “Haplotype M”, with M1 carrying all the listed mutations, and M2 carrying all but the last two mutations. The clones with no amino acid changes from the sexual transcripts (ie. sexual-like) are named “Haplotype S”. The prematurely stopped isoforms do not contain the S574 residue and are marked “NA” in the table.

## Sexual



## Asexual



**Figure 5.2.3 – Schematic of currently identified full-length cDNA haplotypes.**

### 5.2.3 Transcription levels of the alternative isoforms

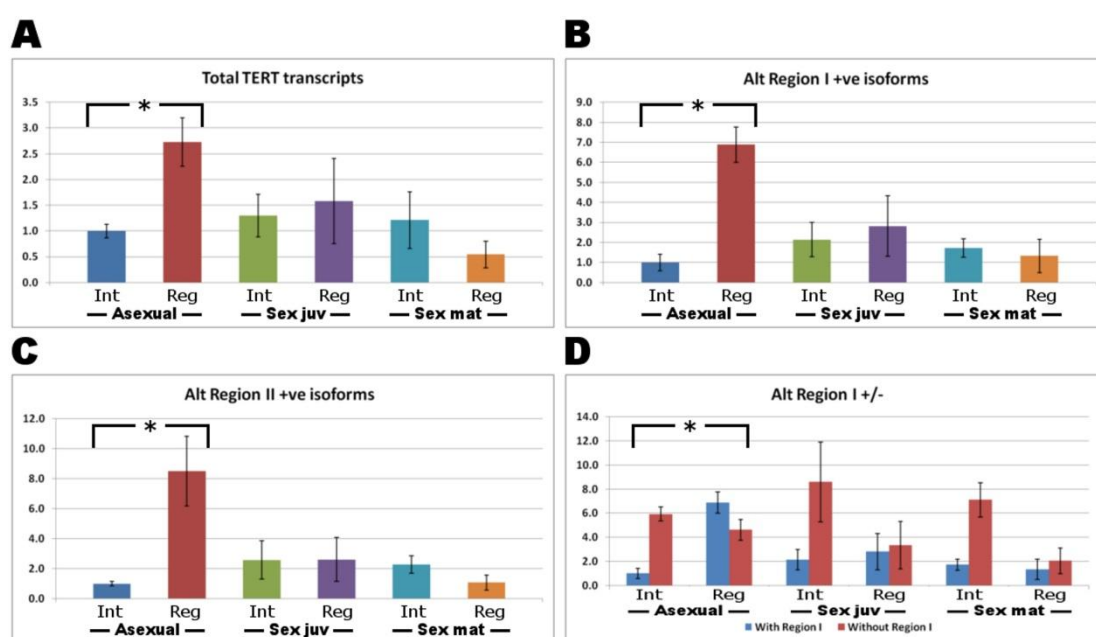
All experiments in this section were done in triplicate, on batches of five worms per replicate. As shown in Figure 5.2.4a, the total copy number of all *Smed\_Tert* transcripts showed an average of 2.73 fold increase ( $p=0.0182$ , T-test, two tailed, unequal variance) in asexual samples during regeneration. This pattern was not observed in the sexual strain, with no significant changes in the juvenile (1.22 fold increase,  $p=0.635$ ) and mature (2.22 fold decrease,  $p=0.160$ ) samples.

Enrichment of Alt Region-containing isoforms in the asexual strain was observed during regeneration. The copy number of the Alt Region I-positive isoforms (see Figure 5.2.4b) increased by 6.89 fold ( $p=0.00237$ ) and the Alt Region II-positive isoforms (Figure 5.2.4c) increased by 8.50 fold ( $p=0.0300$ ). The copy number of these regions in the sexual juveniles did not change significantly (1.32 fold increase,  $p=0.542$  and 1.01 fold increase,  $p=0.985$  for Alt Region I and II, respectively), while a significant decrease was observed in the mature sexual samples in Alt Region II (2.14

fold decrease,  $p=0.0513$ ) but not Alt Region I (1.30 fold decrease,  $p=0.519$ ).

The copy number of the Alt Region I-negative isoforms decreased in all analysed samples by an average factor of 1.29, 2.57 and 3.47 ( $p=0.0998$ , 0.0927 and 0.00943) in asexual, sexual juvenile, and sexual mature, respectively. When analysed together with the Alt Region I-positive copy numbers (see Figure 5.2.4d), the positive/negative ratio of this region increased in all samples during regeneration by an average factor of 8.86, 3.71 and 2.54 ( $p=0.0231$ , 0.0592 and 0.203), respectively.

Various designs for the Alt Region II-negative primer set have failed to produce enough signal for detection, despite the use of extra template (a maximum of 100ng per 25 $\mu$ l reaction was tried).



**Figure 5.2.4 – Quantitative PCR analysis of the transcription level of** (A) the total *Smed\_Tert* transcripts, (B) isoforms containing Alt Region I, (C) isoforms containing Alt Region II, and (D) the isoforms with and without Alt Region I in intact and regenerating samples of both strains.

Error bars display  $\pm 1$  standard deviation. juv: juvenile; mat: mature. Three replicates of batches of 5 worms each were analysed in each sample, asterisks indicate groups with p-values < 0.03.

## **5.3 Discussion**

### **5.3.1 Possible functions of *Smed\_Tert* splicing isoforms**

A unique feature of *Tert* in *S. mediterranea* is the presence of an alternative splicing site in the conserved TRBD domain, in which splicing variations have not so far been discovered in other organisms. Previous studies using experimentally mutated or naturally occurring mutants in this region (Friedman and Cech, 1999, Lai et al., 2001, Yamaguchi et al., 2005, Xin et al., 2007b) have shown the importance of this region for telomerase activity and association of this region with susceptibility to acute myeloid leukaemia. Since the RNA subunit of telomerase has not been identified in this organism, it is not currently possible to study the functions of the splicing variants by *in vitro* reconstruction. Nevertheless, the increase in the positive-negative ratio of Alt Region I during regeneration does suggest the coincidental enrichment of full-TRBD isoforms with the enhancement of telomerase activity seen in the TRAP assay. This enrichment occurs in regenerating samples of both strains, suggesting a splicing regulation of telomerase in the stem cells. With preserved N-terminal and C-terminal sequences, it is possible that the Isoform 2, which carries a truncated TRBD and a complete RT domain, is capable of being recruited to the single stranded telomeres by protein-protein interactions, but has no telomere maintenance activities due to the absence of RNA template. Therefore the Isoform 2 may compete with the full-length Isoform 1 in telomere binding and exhibit an inhibitory effect on telomere maintenance.

The premature stop codon in isoforms lacking Alt Region II (Isoform 3 and 4) is 30bp upstream of the 3' splice junction, which is shorter than the 50bp distance for the NMD pathway suggested by the previous chicken study (Amor et al., 2010). It is uncertain whether these isoforms are stable or subjects of the NMD pathway. Failure of the primer sets to amplify the isoforms lacking this region may be due to the difficulty of designing primers across the splice boundary. Comparing the fold increase in the expression level between total *Smed\_Tert* and Alt Region II-positive isoforms (Isoform 1 and 2) during regeneration in asexual samples, Isoform 1 and 2 increased by 8.50 fold while the total *Smed\_Tert* only increased by 2.73 fold. Therefore it is likely that the proportion of Isoform 3 and 4 in the total transcript is not negligible. If these isoforms are translated, the truncated RT domain and the absence of constitutive C-terminal structure in these proteins suggest they are non-catalytic, and may also be non-telomeric. They may function similarly to the previously studied human TERT which modulates Wnt dependent genes by acting as a cofactor of the  $\beta$ -catenin transcriptional complex in an RT-independent manner (Choi et al., 2008, Park et al., 2009). If these isoforms are targets of the NMD pathway, cells may use the splicing of this region as a way to tag specific isoforms for degradation. This mechanism may be used for controlling the ratio of Isoform 1 and 2, which affects telomere maintenance. With the *Smg1* homolog identified (Cristina Gonzalez-Estevez, unpublished) it is possible to conduct RNAi against the NMD pathway and observe changes in the isoform ratios. It is hypothesised that the enhancement of telomerase activity can be accomplished by shifting the splicing of Alt Region I-positive isoforms towards Isoform 1 (increasing the active form), and the splicing of Alt Region I-negative isoforms towards Isoform 4 (reducing competitive

inhibitors). In order to confirm this hypothesis, further experiments are needed to measure the transcription of individual isoforms.

### 5.3.2 Post-transcriptional splicing control of telomere length dynamics

Although the current data is not sufficient to show the abundance of individual isoforms, it can still provide a mechanical explanation to the telomere and telomerase dynamics observed in Chapter 3. The similar TRAP activities in intact samples of both strains correlate with the similar Alt Region I positive/negative ratios between these samples. During regeneration, increase in the Alt Region I ratio coincides with the enhanced *in vitro* TRAP activities, this increase is conserved between the strains by lowering the transcription of Alt Region I-negative isoforms, and may serve to prevent rapid telomere attrition. In asexuals the decrease in the Alt Region I-negative isoforms is accompanied by the up-regulation of Alt Region I-positive isoforms which does not occur in the sexuals, resulting in a greater difference in the positive/negative ratio between the strains. The fainter TRAP band in the sexual regenerating samples may be due to the lower fold of increase in the positive/negative ratio, together with the lower total *Smed\_Tert* transcription level compared to the asexual samples. The telomere length reset in the asexual strain during regeneration coincides nicely with the transcriptional and post-transcriptional *Smed\_Tert* up-regulation. Whereas the lack of Alt Region I-positive up-regulation in the sexuals may result in insufficient telomere maintenance and general telomere erosion after regeneration, despite the reduction in Alt Region I-negative isoforms.

The significant decrease in the Alt Region II-positive isoforms in regenerating mature sexuals further supports the observation from the *Smed\_Tert* in-situ hybridisation, which suggests that gamete-producing cells hold the majority of telomerase activity in sexual worms. This decrease was not observed in the juvenile sexuals, which may be due to the smaller proportion of proliferating germ cells in the body comparing to the mature worms, and a higher proportion of telomerase activity in soma during growth. The *Smed\_Tert* expression in regenerating mature sexuals is generally lower than that in regenerating juveniles, suggesting a down regulation of *Smed\_Tert* in soma after reaching maturity.

### 5.3.3 Allelic variations of asexual *Smed\_Tert*

Three *Smed\_Tert* haplotypes have been identified in the 15 RT-PCR clones derived from one individual asexual worm. Haplotype S is the sexual-like version of the transcript, with identical amino acid composition to the sexual *Smed\_Tert*. Haplotype M1 contains SNPs, among which some results in polymorphisms at the amino acid level. Haplotype M2 appears to be a mixture between S and M1, most possibly as a result of a single crossover event between G837 and A/T1312. It can be expected that another haplotype, M3, should also be produced by this crossover, however this haplotype was not identified with the limited number of sequenced clones.



**Figure 5.3.1 – Possible crossover event suggested by the three haplotypes currently identified.**

Mutations in *Smed\_Tert* have not been identified in the sexual clones. This suggests that Haplotype M1 arose from spontaneous random mutations after the branching of the asexual lineage from the common ancestor of the two strains. Alternatively, both Haplotype S and M1 may be possessed by the ancestor, and preserved by the asexual strain while the sexual strain abandoned M1. The asexual strain is in theory less capable of removing mutations in the genome due to the lack of meiotic recombination. This idea, advanced from the original Muller's ratchet (Muller, 1964), predicts that allelic variations of a gene may be tolerated if the mutation does not affect the fitness of the stem cell within a population. The M haplotypes consist of all splicing variants, suggesting they are stably transcribed. Although the total number of sequenced clones (n = 15) is not enough to make a definite conclusion, no mutations, including silent mutations, were found in sexual-like *Smed\_Tert* clones, supporting little recombination between the mutants and the wild type allele. Of the five identified mutation sites, two of those are silent mutations (T663C and T1740G for S215 and S574, respectively), two result in possible interchangeable residue variations (CA207-208GG and C865G for I63V and L282V, respectively), and one may have consequences in protein properties (A1340T for K440I). The K440I site locates at the beginning of the Motif D (see aa.885 in Appendix A) of the RT domain, in proximity to a previously studied human mutation site (K902N, end of Motif D) which results in loss of telomerase function and haploid insufficiency in dyskeratosis congenita patients (Armanios et al., 2005). It is not certain whether Haplotype M1 exist in all stem cells, or a sub-population of these cells. Effects of these mutations can be studied in the



future by *in vitro* reconstruction when the telomerase RNA subunit is identified.

## CHAPTER 6: UNFINISHED PROJECTS

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### 6.1 Introductions

#### 6.1.1 Discovery and characterisation of *Pot1* homolog in *S.*

##### *mediterranea*

The full-length transcript of *Smed\_Pot1* (Protection of telomeres – 1) has been recently identified by TblastN and RACE. This protein is featured by the presence of at least one oligonucleotide/oligosaccharide binding fold (OB fold) which confers its affinity to single-stranded telomeric sequence (Horvath et al., 1998). POT1 is conserved between fission yeast and humans in sequence, in its ability to bind telomeric 3' overhang (Baumann and Cech, 2001) and in its role in maintaining the terminal loop structure and shielding telomeres from ATR-mediated DNA repair machinery (Guo et al., 2007). Similar to its structural homolog in budding yeast, CDC13, POT1 also determines the length of 3' overhang by controlling the access of telomerase (Kelleher et al., 2005, Veldman et al., 2004) and C-strand resectioning nucleases to telomeres (Garvik et al., 1995, Hockemeyer et al., 2005). Inhibition or deletion phenotypes of POT1 differ between organisms. Deletion in *S. pombe* leads to rapid telomere loss, telomeric fusion and cell death (Baumann and Cech, 2001), similar to the phenotypes observed in the plant *Arabidopsis thaliana* after expression of a dominant negative allele of *Pot2* (one of the POT1 orthologs) (Shakirov et al., 2005). In contrast, RNAi knockdown in human cells causes reduced proliferation, telomere lengthening, decreased 3' overhang, randomised 5' terminal nucleotides, and formation of  $\gamma$ H2AX DNA damage foci at telomeres but with only a mild increase in telomere fusion (2-fold, compared to 10-100 fold increase in TRF2 knockdown cells) (Hockemeyer

et al., 2005). Conditional knockout of POT1 in chicken DT40 cells results in cell arrest in G2 phase and increased telomere length, but instead of reduced 3' overhangs, cells with no POT1 expression have increased telomeric single-strand to double-strand ratio (Churikov et al., 2006), possibly due to increased access of telomerase to the open telomere structure and unregulated C-strand degradation. Two of the four identified POT1 homologs in *C. elegans*, CeOB1 and CeOB2, were shown to bind to the G-strand and C-strand (unique to the nematodes) overhang, respectively (Raices et al., 2008). Loss of CeOB1 by gene deletion leads to 3' overhang elongation, likely in a telomerase dependent manner, while deletion of CeOB2 causes heterogeneous lengthening of 5' overhangs which resembles the maintenance by ALT pathway. Together, these results demonstrate the rapid evolving nature of the POT1 gene to suit various requirements in telomere protection and maintenance in each organism.

Since only one homolog of POT1 was identified in *S. mediterranea*, it is hypothesised that the function of this protein is similar to its relatives in other multicellular organisms with a single copy of the gene, such as human and chicken. It should possess 3' overhang specificity, regulate overhang length, prevent ATR-mediated telomere damage signals, and the consequential cell cycle arrest at G2.

In this project, RNAi of *Smed\_Pot1* was performed, and the effects of the gene inhibition were characterised. Expression level of marker genes (Eisenhoffer et al., 2008) for adult somatic stem cells (Category 1 cells, represented by *Smedwi-2* expression) and their differentiated progenies (Category 2 and 3, represented by *NB.21.11e* and *Agat1*, respectively) were measured with qRT-PCR. Metaphase chromosome spreads were

carried out with regenerating *Smed\_Pot1* and *dsRed* RNAi samples (Day 3 post amputation) to observe the effects of the gene inhibition on genome stability and cell proliferation. Various planned experiments will be discussed in the final section of this chapter.

## **6.2 Results**

### **6.2.1 Gene discovery, protein annotation, and inhibition phenotypes of *Smed\_Pot1***

As shown in Figure 6.2.1, a region between G4317-G2569 on the negative strand of Contig v31.002347 encodes a 1371bp transcript which is translated into the 334aa *Smed\_POT1* protein. The site of highest homology with other species is Exon 3, forming the N-terminal OB1 fold of the protein, which was confirmed with the NCBI conserved domain search (Marchler-Bauer et al., 2011) matching the OB1 fold of human POT1 (for sequence alignment see Figure B1 in Appendix). A second OB2 fold was predicted by the Phyre software (Kelley and Sternberg, 2009), showing structural similarity to the OB2 fold of human POT1 (see Figure B2 in Appendix).

The RNAi phenotype of *Smed\_Pot1* consists of an acute effect on the anterior axis, followed by persistent regeneration defects and tissue regression which led to death of the treated samples. All RNAi treated worms decreased in body size throughout the injection regime more rapidly than the *dsRed* controls. A small proportion of the worms lost their head region (from the middle point between the photoreceptors and the pharynx) within 1-4 days from the first injection, and regenerated a small head with normal eyes after 7 days. Following amputation (see Figure

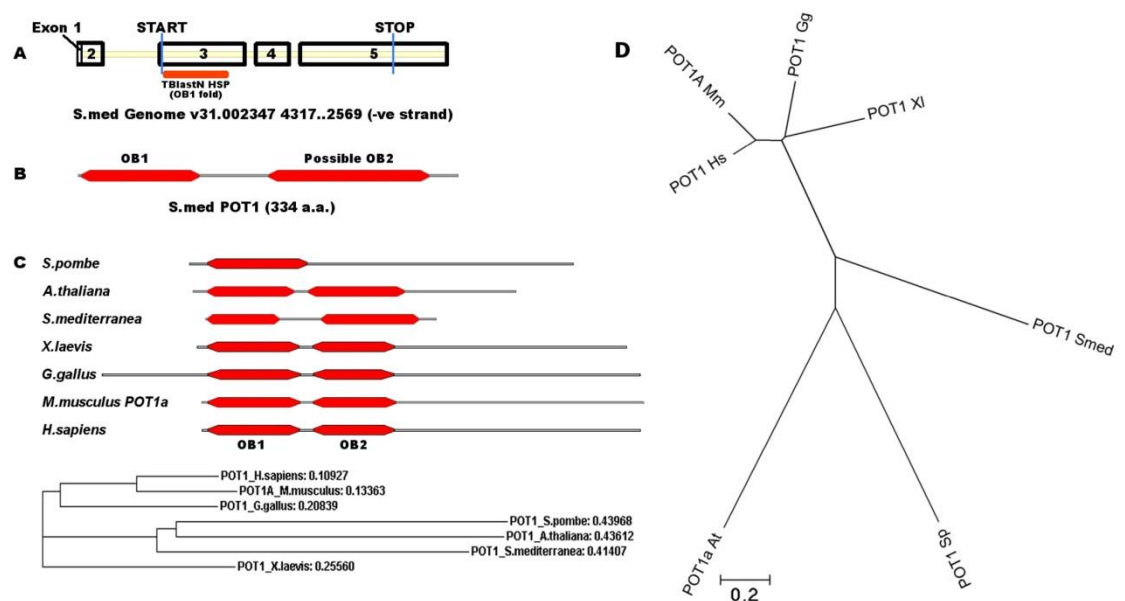
6.2.2c), all samples developed regeneration blastemas which were smaller than those in the controls. Tissue regression occurred at various rates between samples, generally the existing head tissues started to regress after Day 10, and the posterior blastemas after Day 12. All middle pieces regenerated smaller heads compared to the controls, and in some samples these heads started regressing after Day 14. All tail pieces had incomplete head regeneration, some did not form visible eyes and others had eye regression after Day 14. Death began to be observed from Day 14 in the most severe case, followed by other pieces with milder phenotypes at various time points. 78.3% of the pieces died by Day 30 while the remaining reverted back to wild type after the effect of RNAi diminished. The phenotypes and their abundances are listed in Table 6.2.1.

| <b>Time point</b>                 | <b>Type</b>                          | <b>Description</b>                    | <b>Abundance</b>                     |
|-----------------------------------|--------------------------------------|---------------------------------------|--------------------------------------|
| During injection regime           | Rapid body size reductions           | From ~9mm to ~5mm                     | 60/60 (100%)                         |
| 1-4 days from the first injection | Head lysis                           | From between the eyes and the pharynx | 4/60 (6.67%)                         |
| Day 5 post amputation             | Small blastema                       |                                       | 60/60 pieces (100%)                  |
| After Day 10 pa.                  | Existing head regression             | In head pieces                        | 19/20 pieces (95%)                   |
| After Day 12 pa.                  | Posterior blastema regression        | In head and middle pieces             | 40/40 pieces (100%)                  |
| After Day 14 pa.                  | Regression of regenerated small head | In middle and tail pieces             | 11/20 middle (55%), 19/20 tail (95%) |

|                                   |        |                                      |   |
|-----------------------------------|--------|--------------------------------------|---|
| Varies widely,<br>From Day 14 pa. | Death  | Total death counted<br>on Day 30 pa. | 18/20 head (90%),<br>10/20 middle<br>(50%), 19/20 tail<br>(95%) |
| After Day 30 pa.                  | Rescue | Reverted back to wild<br>type.       | 1/20 head (10%),<br>10/20 middle<br>(50%), 1/20 tail<br>(5%)    |

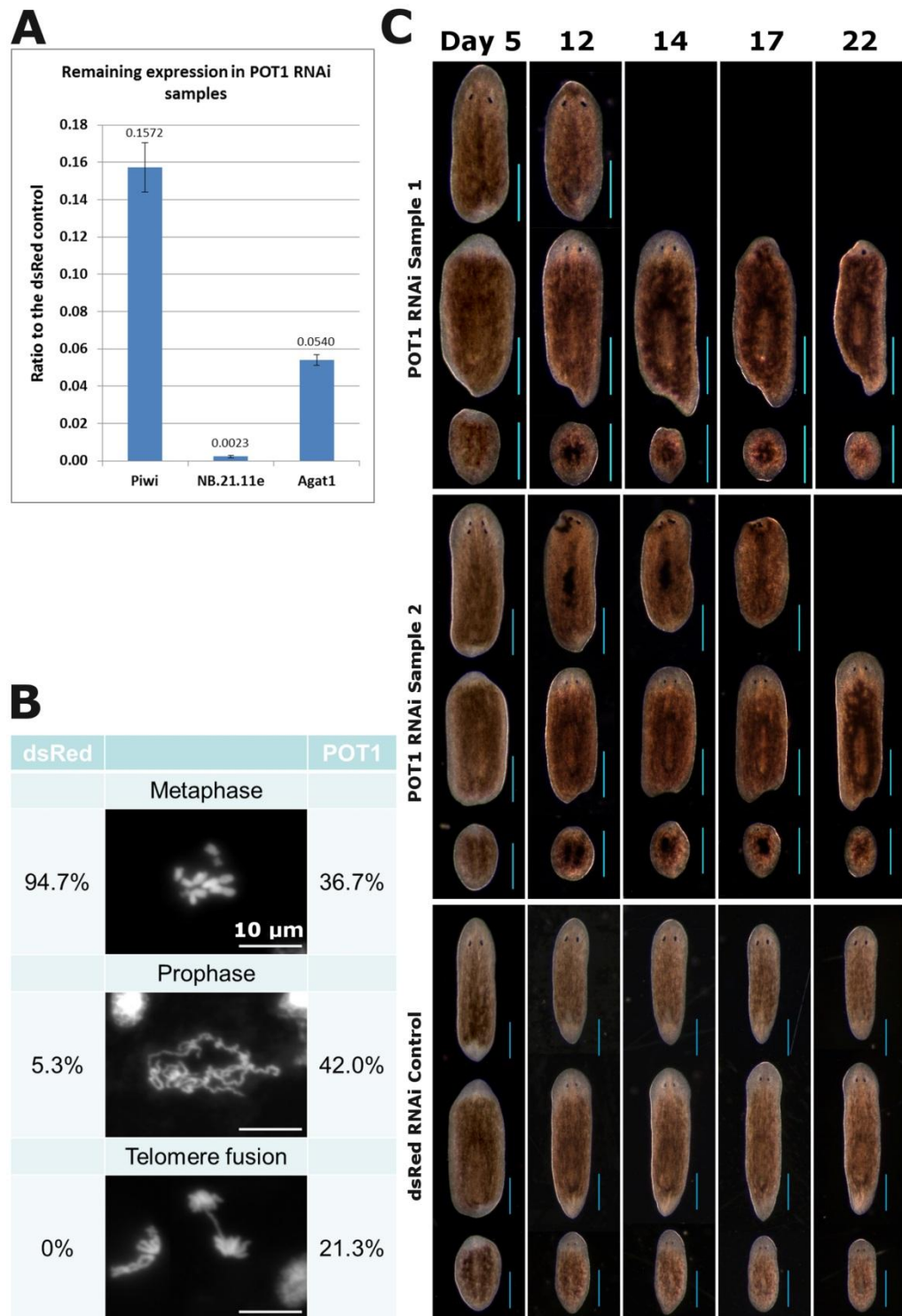
**Table 6.2.1 – *Smed\_Pot1* RNAi phenotypes and their penetrance.**

qRT-PCR on category marker expression (done in three replicates on batches of five worms each) displayed an average decrease of 84.3% in *Smedwi-2*, 99.8% in *NB.21.11e*, and 94.6% in *Agat1* (s.d.= 1.33%, 0.0534%, and 0.299%, respectively) in RNAi samples on Day 17 post amputation, as shown in Figure 6.2.2a. The metaphase chromosome spread (see Figure 6.2.2b) in *Smed\_Pot1* RNAi samples showed a reduction in mitotic figure, accompanied by an increased ratio of prophase cells (8 in 20 counted cells). In contrast, metaphase chromosomes were more abundant in the dsRed controls, with 1 prophase cell in 50 cells totally counted.



**Figure 6.2.1 – Gene and protein annotation of Smed\_POT1. (A-B)**

The 331aa protein is translated from a 1369bp transcript which is mapped to the genomic Contig v31.002347. The OB1 fold was mapped with the NCBI conserved domain search, while the OB2 fold was predicted with Phyre online software. **(C)** Multi-species alignment and phylogram of POT1 proteins, showing their relative sizes and domain distributions (protein sequences obtained from NCBI RefSeq protein database). **(D)** The evolutionary tree of POT1 computed using the Minimum Evolution method with MEGA5 software. The branch lengths are drawn to scale, in the units of the number of amino acid substitutions per site.



**Figure 6.2.2 – Current *Smed\_Pot1* phenotypic characterisations.**

**(A)** Expression levels of category cell markers in the *Smed\_Pot1* RNAi treated samples on Day 17 post-amputation, shown as ratios to the levels in the *dsRed* control samples (n = 3). **(B)** Metaphase chromosome spread of 72hr regenerating sexual samples. Worms treated with *Smed\_Pot1* RNAi



are featured by low mitotic figures and a high abundance of prophase chromosomes and telomere fusions (n = 150). **(C)** Regeneration defects and tissue regressions in *Smed\_Pot1* RNAi samples. Time points are shown as days post-amputation. Scale bar = 0.5mm.

## **6.3 Discussion**

### **6.3.1 *Smed\_Pot1* RNAi may cause G2 arrests and gradual stem cell depletions**

As reviewed in the introduction, POT1 protein evolves rapidly between species. *S. mediterranea* has a single copy of the *Pot1* gene, and the protein is small and compact compared to other organisms in the alignment. The *Smed\_POT1* is unique in a way that the two OB folds are located at either end of the protein, while these folds are commonly found in the N-terminal region in other organisms. The ability of regenerating worms to grow missing heads and eyes suggests that the *Smed\_Pot1* RNAi worms are still able to induce cell proliferation and differentiation, possibly due to residual *Smed\_POT1* protein binding at the telomere ends of proliferating cells which prevent the formation of DNA damage signals. The smaller size of the regenerated tissues correlates with the low mitotic figure observed in the metaphase chromosome spread on Day 3 post amputation, which indicates that some stem cells were starting to lose their proliferative capacity. The high abundance of prophase cells suggests a cell cycle arrest at late G2, when chromatin begins to condense. It is likely that as the experiment progressed, residual *Smed\_POT1* protein in cells began to deplete, resulting in more cellular arrest and apoptosis. This progressive reduction of proliferative stem cells led to inability to maintain existing tissues, causing regression which is more visible in body regions

with high turnover rates. The Category 2 cells have been previously shown as a population of transient and short-living stem cell progeny which differentiate into Category 3 cells approximately four days after division from the stem cells (Eisenhoffer et al., 2008). Prompt removal of stem cells by irradiation causes down regulation of Category 2 markers in two days, and Category 3 markers in three to four days. Since both Category 2 and 3 cells are concentrated in an area anterior to the photoreceptors, regression of this area in *Smed\_Pot1* RNAi samples was most prominent. The category marker expressions on Day 17 post amputation also indicate a failure in the homeostasis of category cells. 15.7% of *Smedwi-2* stem cell marker expression remained in the *Smed\_Pot1* RNAi samples, these remaining cells were either quiescent or senescent, and could not give rise to the short-living Category 2 cell population, as indicated by the 0.23% remaining *NB21.11e* expression. The lack of Category 2 cells resulted in depletion of the relatively long-living Category 3 cells, with 5.4% remaining marker expression.

Several experiments are currently in plan to fully characterise the *Smed\_Pot1* RNAi phenotype. Samples at various time points post amputation should be analysed with FACS to look for the accumulation of 4n DNA cells, which provides supporting evidence for G2 arrest. The arrest and apoptosis are most likely to be mediated by ATR, and therefore the phenotypes should be alleviated by RNAi against ATR. The samples should also be stained with the anti-phosphorylated histone H3 serine 10 antibody which can provide evidence for reduced proliferation. Levels of activated caspase 3 should be measured in the samples as evidence for increased cell death. Whole mount in-situ hybridisation should be done with category markers to confirm the results of the qRT-PCR.

Since the nucleoprotein complex structure at the telomere has not been previously studied in planarians, it is important to understand whether *Smed\_Pot1* inhibition causes DNA damage signals specifically at the telomeres. Previous western experiments have shown that the anti-γH2AX serine 139 antibody (Cell Signalling) have failed to produce signals with irradiated *S. mediterranea* (data not shown). Antibodies against other phosphorylated serines are yet to be tested. The length of 3' overhang should also be measured at different time points using non-denaturing TRF analysis. As contradictory results in the overhang dynamics of *Pot1* inhibited cells have been found in different organisms, it is intriguing to see how POT1 functions *in vivo* in an invertebrate model with canonical (TTAGGG)<sub>n</sub> telomere repeats and with cells in their natural niches.

## CHAPTER 7: CONCLUSIONS

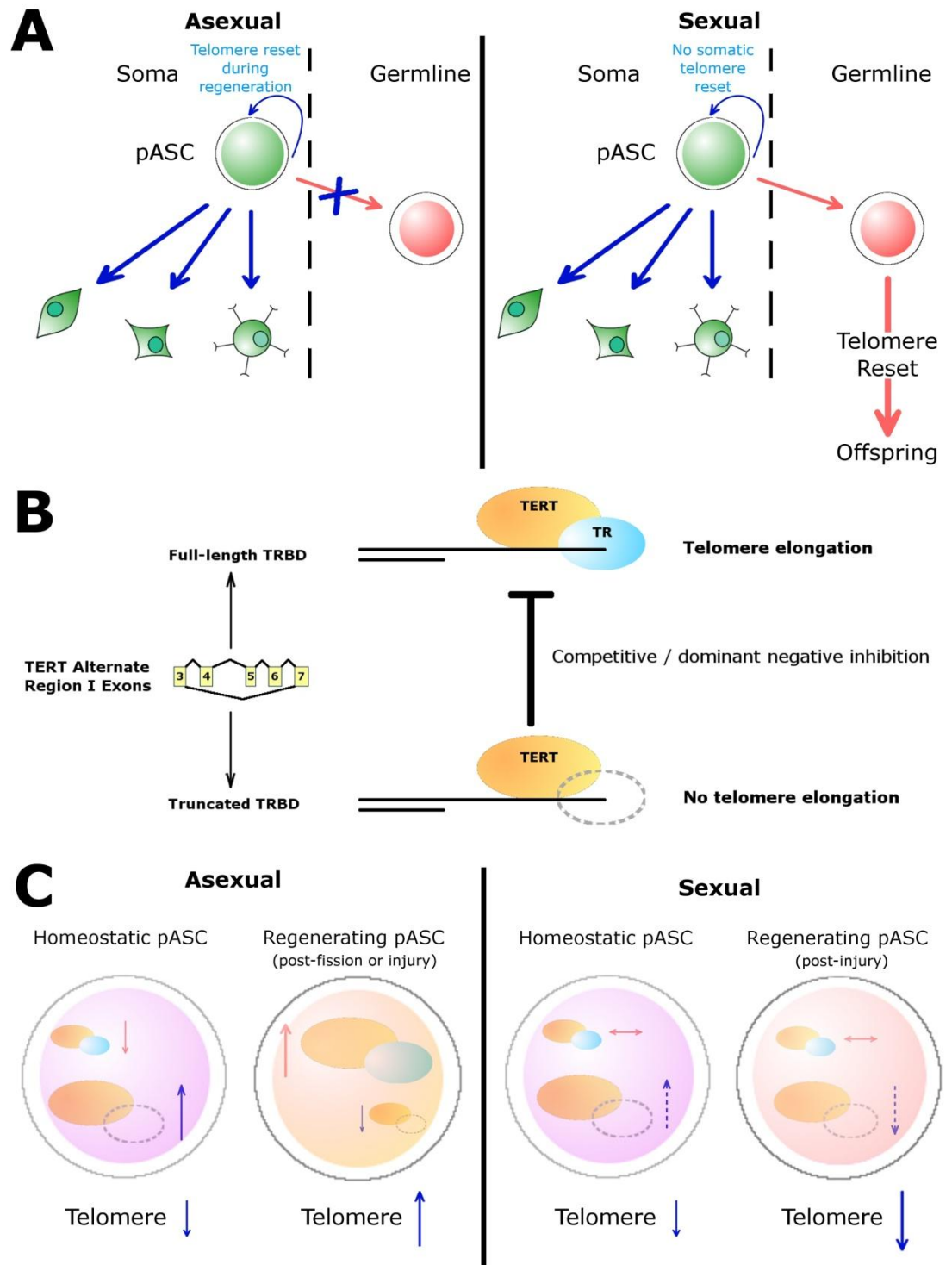
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### **7.1 Telomerase activity in adult stem cells is adapted to an immortal life history in obligate asexual *S. mediterranea***

As discussed in Chapter 3, the asexual strain of *S. mediterranea* replenishes its somatic telomeres through periodic fission and regeneration. The RNAi experiment in Chapter 4 has confirmed that this reset is telomerase dependent, and the target length for the reset may be determined by a stem cell population with the longest telomeres. Results from Chapter 5 indicate that the reset involves transcriptional up-regulation of the *Smed\_Tert* gene, as well as post-transcriptional splicing regulation, which result in enrichment of the full-length *Smed\_Tert* isoform and down regulation of isoforms lacking intact TRBD regions. In contrast, this regeneration-associated somatic reset is absent in the sexual strain, which maintains its telomeres via passage through the germline, a common mechanism in multicellular organisms (Figure 7.1.1A and C).

*Smed\_Tert* isoforms containing truncated TRBD are predominant in intact worms and decrease during regeneration. This decrease correlates with the enhancement of TRAP activity in these samples, suggesting a possible inhibitory property of these isoforms. Since mutations in TRBD in human and yeast impair interaction with the telomerase RNA subunit and holoenzyme dimerisation, these isoforms are not likely to possess telomere-maintaining function, but can possibly still associate with telomeres via protein-protein interactions between the POT1/TPP1 complex and/or CST complex with their N and C-terminal regions, and compete with the functional full-length isoform (Figure 7.1.1B).

Unlike a previous observation in colonial ascidian *Diplosoma listerianum* which requires occasional sexual reproduction to achieve rejuvenation (Sköld et al., 2011), asexual *S. mediterranea* has evolved a mechanism that makes its somatic stem cells effective units of inheritance. The reset mechanism prevents telomere depletion caused by the end replication problem over an evolutionary timescale, and demonstrates the adaptation to the agametic asexual life mode, including immortalisation of somatic stem cells in their native niche. Further study is required to understand how the regeneration-induced *Smed\_Tert* transcription and splicing is regulated, and how the regulation differs between the strains; this will involve comparisons between the promoter region of the gene, as well as exonic/intronic splicing regulatory motifs throughout the gene. This plan requires a sequenced genome of the asexual strain, which is currently not available. An alternative approach is to isolate the entire genomic region surrounding the *Smed\_Tert* gene (approximately 20kb) by Transformation associated recombination (TAR) cloning, followed by sequencing of the clones.



**Figure 7.1.1 – Splicing control of telomerase activity and telomere reset in pASCs of asexual and sexual strains. (A)** Sexual germline is derived from somatic pASCs. Unlike the telomere reset in asexual pASCs during regeneration, telomeres in the sexual strain are reset in the germline, during gametogenesis or embryogenesis. **(B)** Alternate splicings

of *Smed-Tert* alternate region I produce isoforms with truncated TRBD, which is thought to cause failure in interactions with the telomerase RNA subunit (TR) and a consequent lack of telomerase activity. Reduction in the TRBD negative isoforms during regeneration correlates with the increase in telomerase activity, indicating that these isoforms may act as competitive or dominant negative inhibitors of telomerase. **(C)** Comparison of the isoform expression levels between homeostatic and regenerating pASCs in both strains. Significant up-regulation of the intact/truncated TRBD ratio during regeneration coincides with enhanced TRAP activity and telomere length reset in the asexual strain. While a slight increase in the ratio in the sexual regenerating pASCs is not sufficient to prevent telomere erosions during rapid cell divisions.

## 7.2 *S. mediterranea* as an *in vivo* model for telomere biology in adult multicellular organisms

Although the telomeric regions in *S. mediterranea* have yet to be cloned and sequenced, the presence of (TTAGGG)<sub>n</sub> arrays in the genome, together with the sensitivity of the (TTAGGG)<sub>n</sub> signals to Bal-31 in the TRF analysis and its decrease in length in *Smed\_Tert* RNAi treated samples, have indicated that *S. mediterranea* utilises the same telomeric repeat unit as previously found in *Polycelis tenuis*. This repeat unit is commonly possessed by organisms across the eukaryotic kingdom, such as the filamentous fungi *Neurospora crassa* (Schechtman, 1990), acellular slime moulds *Didymium iridis* and *Physarum polycephalum* (Forney et al., 1987), kinetoplastid protozoan *Trypanosoma brucei* (Blackburn and Challoner, 1984, Van der Ploeg et al., 1984), sponge species (Porifera) (Sakai et al., 2007), annelid worms (Vitturi et al., 2000, Jha et al., 1995), molluscs (Wang and Guo, 2001, Sakai et al., 2005, Gallardo-Escarate et

al., 2005), velvet worms (Onychophora) (Vitkova et al., 2005), and among vertebrate species (Meyne et al., 1989), and is therefore likely to be the ancestral telomeric sequence of the eukaryotes. The presence of telomerase and its vertebrate-like 6bp processivity indicates a canonical telomere maintenance mechanism in planarians. Although it is not certain if planarians possess 5' telomeric overhangs similar to *C. elegans*, the single copy of *Pot1* in *S. mediterranea* suggests a single type of overhang, which is most likely to be the common 3' stretches. In addition to TERT and POT1, bioinformatic analysis has also preliminarily suggested the presence of various telomere associated protein homologs including TRF2, RAP1, Tankyrase, BLM helicase, DNA-PK and the checkpoint-activating ATM and ATR kinases (details listed in Table 7.1). These genes have not been studied in planarians. Other shelterin complex proteins may be present but cannot be identified with the current transcriptome assembly, it is expected that the universally conserved OB fold containing CST complex (Cdc13-Stn1-Ten1 in budding yeast (Gao et al., 2007), CTC1-STN1-TEN1 in mammals (Miyake et al., 2009) and plants (Surovtseva et al., 2009)), which binds to the single stranded telomeric overhangs, should also be present in *S. mediterranea*.

| <b>Protein</b> | <b>EST name</b>         | <b>Top hit organism</b>              | <b>E Value</b> |
|----------------|-------------------------|--------------------------------------|----------------|
| TRF2           | lcl_AAA.454ESTABI.7659  | <i>Taeniopygia guttata</i>           | 0.083          |
| RAP1           | lcl_AAA.454ESTABI.16947 | <i>Strongylocentrotus purpuratus</i> | 4e-13          |
| ATM            | lcl_AAA.454ESTABI.12461 | <i>Ciona intestinalis</i>            | 3e-56          |



|                      |                         |                                       |        |
|----------------------|-------------------------|---------------------------------------|--------|
| ATR                  | ec1.14936.001           | <i>Taeniopygia guttata</i>            | 4e-44  |
|                      | v31.013442_4105..19586  | <i>Callithrix jacchus</i>             | 2e-08  |
| Tankyrase            | lcl_AAA.454ESTABI.21226 | <i>Saccoglossus kowalevskii</i>       | 5e-138 |
| DNA-PK               | lcl_AAA.454ESTABI.10238 | <i>Polysphondylium pallidum</i> PN500 | 8e-28  |
|                      | lcl_AAA.454ESTABI.12021 | <i>Canis lupus familiaris</i>         | 7e-17  |
| BLM (Bloom syndrome) | lcl_AAA.454ESTABI.21054 | <i>Acyrtosiphon pisum</i>             | 3e-165 |

**Table 7.1 – Preliminarily identified telomere-associated protein homologs.**

Models commonly used in telomere studies consist of *ex vivo* cell cultures of higher vertebrates in the absence of native cellular environments, *in vivo* studies in higher vertebrates (mouse and chicken) which are difficult to characterise in adults, or simple *in vivo* models (yeasts, ciliates, and nematodes) which have diverged telomeric repeats or structures. *S. mediterranea* possesses the (TTAGGG)<sub>n</sub> telomere tandem repeat maintained by the telomerase holoenzyme. Studies in this organism can take advantages of these canonical properties, as well as the native *in vivo* adult stem cell niches in which genes are easily manipulable by RNAi, and whole body phenotypes can be easily analysed and monitored by the improving molecular techniques which are being made available to planarians. Distribution and number of proliferating cells in dysfunctional telomere backgrounds can be observed by the phospho-histone H3S10 immunohistochemistry, cells with proliferative capacities can be monitored by in situ hybridisations and qPCR using various markers such as PCNA and

Category 1 genes, and their developmental progenies can be assessed with Category 2, 3 and other tissue specific markers. The improving understanding of cell lineages and cell type specific genes, together with the ability of adult worms to reset developmental pathways during regeneration, make this planarian species a robust tool for studying the developmental functions of telomere associated genes, such as the role of TERT in Wnt regulation. Although the *Smed\_Tert* inhibition by RNAi was not sufficient to induce developmental defects, it is hoped that a transgenic conditional knockout approach can be adopted in the future.

In addition to the advantages provided by the simplicity of whole body characterisations, this organism also possesses complex reproductive cells with high telomere maintenance activities, which can be used for understanding how telomerase can be regulated in the germline. Current results from the expression levels of *Smed\_Tert* isoforms suggest an important role of splicing control in the regulation of telomere maintenance in both sexual and asexual strains. Whether this control involves dominant negative isoform(s) and/or the nonsense-mediated decay pathway requires further experiments.

In this PhD project several common techniques in telomere studies have been applied and optimised in this organism. Telomere length was measured using TRF and qPCR, telomerase activities were assayed using TRAP, and cellular consequences of *Smed\_Pot1* RNAi was observed by metaphase chromosome spreads (previously optimised by (Lamatsch et al., 1998)). Various techniques can be optimised for future telomere studies. Proportions of proliferating and G2 arrested stem cells in the whole body can be measured with FACS by their DNA contents, and

analysed in greater depth after sorting. FACS with double-labelled dissociated cells can also be useful for studying the cell-type specificity of telomere lengths. *In vitro* telomerase reconstruction is needed for studying the functions of splicing and mutational variants of *Smed\_Tert*, it is therefore necessary to identify the RNA subunit of telomerase (TERC) in this species. The universally conserved secondary structure of the RNA and the presence of telomeric repeats should allow bioinformatic predictions of possible sequences from the genomic and transcriptomic database.

Furthermore, development of transgenic epitope tagging, gene deletion and over-expression are necessary for complete functional and quantitative studies of proteins in planarians. An electroporation-based method for transposase-mediated genome integration and stable expression of an extrinsic gene has been previously applied to a neighbouring species *Girardia tigrina* with a *Pax6*-specific promoter (Gonzalez-Estevez et al., 2003). However application of various approaches, including electroporation and viral transfection, has yet to produce expressed transgenes in *S. mediterranea*. Identification of strong and ubiquitous promoters for this species may be a major current obstacle (Cristina Gonzalez-Estevez and Sunir Malla, personal communication).

As reviewed in Chapter 1, oxidative stress can cause sudden and massive loss of telomeric sequences. However whether the natural accumulation of ROS is sufficient to erode telomeres *in vivo* requires experimental evidence. The significance of ROS in the maintenance of the natural stem cell pool and whole body ageing can be assessed with comparative studies between the immortal asexual strain and ageing sexual strain. Biochemical assays for activities of antioxidants such as catalase and superoxide

dismutase have been previously applied to planarians (Li, 2008), and can be used to test whether the asexual strain has a higher level of defence against oxidative damages. Genes encoding for these antioxidants can be inhibited and their effects on telomere length can be observed. A more sensitive method for telomere length measurement may be required to see these effects. Recent development of the universal Single telomere length analysis (STELA) technique may provide sufficient resolution for this purpose, however the maximum detection length of this method seems to be limited by the processivity of the DNA polymerase in the PCR reaction, which was found to be approximately 8kb (Bendix et al., 2010). *Smed\_Tert* inhibition has created a line of asexual *S. mediterranea* with a stable mean telomere of less than 10kb (shown in the Week 57 sample of Figure 4.2.4b), which makes the application of this technique a possibility.

### **7.3 *S. mediterranea* as a model for ageing in multicellular organisms**

One of the major steps towards adaptation to the asexual life history is the ability to prevent senescence of the soma. The most prominent difference in telomere dynamics between the sexual and asexual strains occurs during regeneration. Decrease in the transcription of Alt Region I-negative isoforms is observed in both strains, the consequential rise in the positive/negative ratio of the isoforms containing this region may provide a basal protection against massive telomere erosion during rapid cell divisions. The up-regulation of total *Smed\_Tert* transcription in somatic stem cells during regeneration is unique to the asexual strain, and therefore should play an essential role in immortalising the soma. On the other hand, the age-related telomere erosion and lack of length reset in the sexual strain indicates its mortal nature, which is also suggested by the

limited life spans of closely related sexual planarian species previously studied (Child, 1914, Haranghy and Balázs, 1964). Genes of which regulation contributes to the fundamental difference in somatic mortality between the sexual and asexual strains may affect the life span of other organisms as well. Previous proteomic work has predicted the association of 2,163 human proteins to human homologs of known longevity-related proteins in yeast and invertebrates (Bell et al., 2009). Some of these associated proteins also showed effects on longevity in *C. elegans*, showing a conservation of the protein network which controls ageing in human and invertebrates. Recent improvement in transcriptomic sequencing using a combination of two next generation sequencing platforms has allowed a complete bird's-eye view of gene regulation between samples (Blythe et al., 2010). It is now possible to discover ageing related genes with an approach alternative to prediction based on protein associations, by direct comparison between the transcriptome of the two strains during regeneration. This PhD project has focused on telomere related genes due to the essentiality of telomere maintenance for immortal soma, however the regulators of cellular maintenance are yet to be fully discovered. Elements regulating the ageing process may also involve germline property specifications, energy metabolism, oxidative damage removal, and DNA damage repair. It is hoped that the result of this transcriptomic approach, using two strains of the same species with opposite somatic mortalities, can be combined with the previous proteomic prediction to produce significant candidates for experimental characterisations.

## APPENDIX A

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### *Multi-species alignment of TERT using ClustalW2*

1 10 20 30 40 50 60  
TERT\_Hs MPRAPRCRAVRSLRSHYREVLPLATFVRRLLGPQG...WRLVQRGDPAAFRALVAQCLVCPVWDARPPPA  
TERT\_Fr .MSITDLSPITLGLRSLYPHVQVLVDFADDIVFREGHKATLIEESDTSHFKSFVRGIFVCFHKLQQVPS  
TERT\_Smed .....  
TERT\_Tt MQKINNINNNKQMLTRKEDLLTVLKQISALKYVS..LYEFLATEKIVQTSSELDTQFQEFLLTTIIASE  
Est2p\_Sc .....MKILFEFIQDKLDIDLQNSTYKEN.....LKCGHFNGLDEILITCFALPNSRKIALPC

70 80 90 100 110 120 130  
TERT\_Hs APSFRQVSCLELVARVLQRLCERGAKNVLAFGFALLDARGGPPEAFTTSVRSYLPNTVTDALRGSGAW  
TERT\_Fr CN...QICTLPELLAFVLNSVKRRKRNRNLAHGYNFQSLAQE.ERDADQFKLQGDVT.QSAAVYHGSGLW  
TERT\_Smed .....  
TERT\_Tt QNLVENYKQYNQPNFSQTLIKQVIDDSIILLGNKQNYVQQIGTTTIGFYVEYENINLSRQTLYSSN..F  
Est2p\_Sc LPG..DLSHKAVIDHCIIYLLTGELYNNVLTFG.....YKIARNEDVNNSLFCHSANVN

GQ

140 150 160 170 180 190 200  
TERT\_Hs GLLRRVGGDDVLVHLIARCLFVVLVAPSCAYQVCGPPLYQLGAATQARPPPHASGPRRLGCFRAWNHSV  
TERT\_Fr RKVSMRLGTDITRYLFESCQSVFVAVPPSCLFQVCGPIYDCFLSATASLGFSLSRGCRERCIGVNSMKR  
TERT\_Smed .....MKLDLGTALLKLCQKFCIYELTVRSQELN.....NNFEFDEKTKTIKNCSS.....  
TERT\_Tt RNLLNIFGEEDFKYFLIDFLVFTKVEQNGYLQVAGVCLNQYFSVQVQKKWKYNFNFMNGKATSNNN...  
Est2p\_Sc VTLLKGAAWKMFHSLVGTYAFVLDLLINYTVIQFNGQFFTQIVGNRCNEPHLPPKVVQRSSSSSAT.....

?

210 220 230 240 250 260 270  
TERT\_Hs REAGVPLGLPAPGARRRGGSASRSLPLPKRPRRGAAPPEPRTTPVGGQSWAHPGTRGPGSDRGFCVVSPAR  
TERT\_Fr RAFNVKR.....YLRKRKTETDQKDEARVCSGKR.....RRVMEEDKVCSETMQDG  
TERT\_Smed .....EIKKRSPYKSSNSYSGEG.....  
TERT\_Tt .....QNNANLSNEKKQENQYIYPEIQ.....RSQIFYCNHMGRE  
Est2p\_Sc .....AAQIKQLTEPVTNKQFLHKLN.....

280 290 300 310 320 330 340  
TERT\_Hs PAEEATSLEGALSGTRHSHPSVGRQHAGPPSTSRPPRPWDTPCPPVYAETKHFLYSSGDKQLRPSFLL  
TERT\_Fr ESGKTTLVQKQPGSKKRSEMEATLLPLEGGPSWRSGTFPPLPPSQSFMRTLGLFYGGGRMRSFLLNRKKK  
TERT\_Smed .....  
TERT\_Tt PGVFKSSFFNYSEIKKGFQKVIQEKLQGRQFINSDKIKPDHPQTIKKTLLKEYQSKNFSQCEERDLFL  
Est2p\_Sc .....INSSFFPYSKILPSSSSIKKLTDLREAFPTNLVKIPQR....

350 360 370 380 390 400 410  
TERT\_Hs SSLRPSLTGARRLVETIFLGRPMPWPGTPRRRLPRLPQRYWQMRPLFLELLGNHAQCFYGVLLKTHCPLRA  
TERT\_Fr TAEGFRKIQGRDLIRIVFFEGVLYLNGLERKPKKLPARRFNMVPLFSQLLRQHRRCFYSRLLQKTCPLVG  
TERT\_Smed .....  
TERT\_Tt EFTEKIVQNFHNIN.....FNYLLKKFCKLPENYQSLKSQVKQIVQSENKAN.....  
Est2p\_Sc .....LKVRLNLTQLKLLKRHKRLNYSILNSICP.....

QFP

420 430 440 450 460 470 480  
TERT\_Hs AVTPAAGVCAREKPGQSVAAPEEDTDPRLVLQLLRQHSSPWQVYGFVRACLRRLVPPGLWGSRHNNERRF  
TERT\_Fr .....IKDAGQAELSQFLPQHCQSHRVYLFVRECLLAVIPQELWGSSEHNRLLY  
TERT\_Smed .....KIVPDSINHYQVFALKSQKCLNLGLDIFLNSLNTSIF  
TERT\_Tt .....QQSCENLFNSLYDTEISYKQITNQLRQIIQNCVFNQLLGAKNFKVF  
Est2p\_Sc .....PLEGTVLDLSHLSRQSPKEKVLKFTIIVILQKLLPQEMFSGSKKNGKI

490 500 510 520 530 540 550  
TERT\_Hs LRNTKKFISLGKHAKLSLQELTWKMSVRDCAWLRRSPGVGCVPAAEHR.LREITAKFLHWLMSVYVVEL  
TERT\_Fr FARVRFLLRSGKFERLSVAELMWKIKVNNCDWLKIS.KTGRVPPSELS.YRTQILGQFLAWLLDGFVVL  
TERT\_Smed .....SKCNTYT.LHELYIYIMSYILFYEFVVIPI  
TERT\_Tt LEKLYEFVQMKRFENQKVLDYICFMDVFDVWFVLDLKNQKFTQKRYISDKRKILGDLIVFIINKI VIPV  
Est2p\_Sc IKNLNLLLSLPLNGYLPFDLSLLKKLRDKDFRWLFIS...DIWFTKHNFENLNQILICFISWLFRLQIPKI

T 1

560 570 580 590 600 610 620  
TERT\_Hs LRSFHYVTETTFQKNRLFFYRKSVWSKLQSIGIRQHLKRQVQLRELSEAQVRQHREARPALLTSLRFIPK  
TERT\_Fr VRACFYATESMGQKNAIRFYRQEVWAKLQDLAFRSHISKQGMVELTPDQVAALPKS...TIISRLRFIPK  
TERT\_Smed LKQNFHWCESPANSSSVVYFTLSQWHEFQKASAYYINRGIIFSGPISIERIKEIKNYNVGTARCLVKSL  
TERT\_Tt LRYNFYITEKHKEGSQIFYYRKPIWKLVSKLTVKLEEE...NLEKVEKLIPEDSFQKYPQGLRIIPK  
Est2p\_Sc IQTFHYCTEIS.STVTIVYFRHDWNKLTTPFIVVEYFKTYLVENNVCRNHNSTYLSN..FNHSMRIIPK



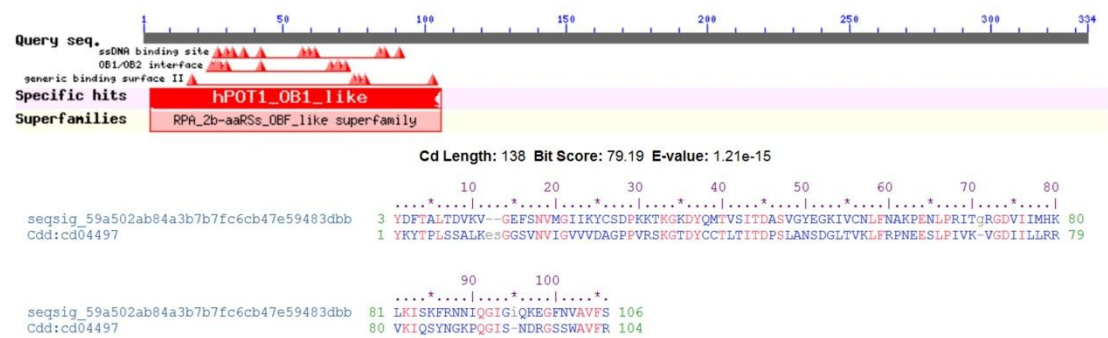
149



**Figure A1 – ClustalW protein sequence alignment of TERT in several distantly related species.** Conserved sequence motifs in *S. mediterranea* were identified by alignment with TERT proteins in *Homo sapiens* (accession number O14746.1), *Fugu rubripes* (AAX59693.1), *Tetrahymena thermophila* (O77448.1), and Est2p in *Saccharomyces cerevisiae* (Q06163.1). Alignment was performed on the EMBL-EBI ClustalW2 (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>) with default parameters.

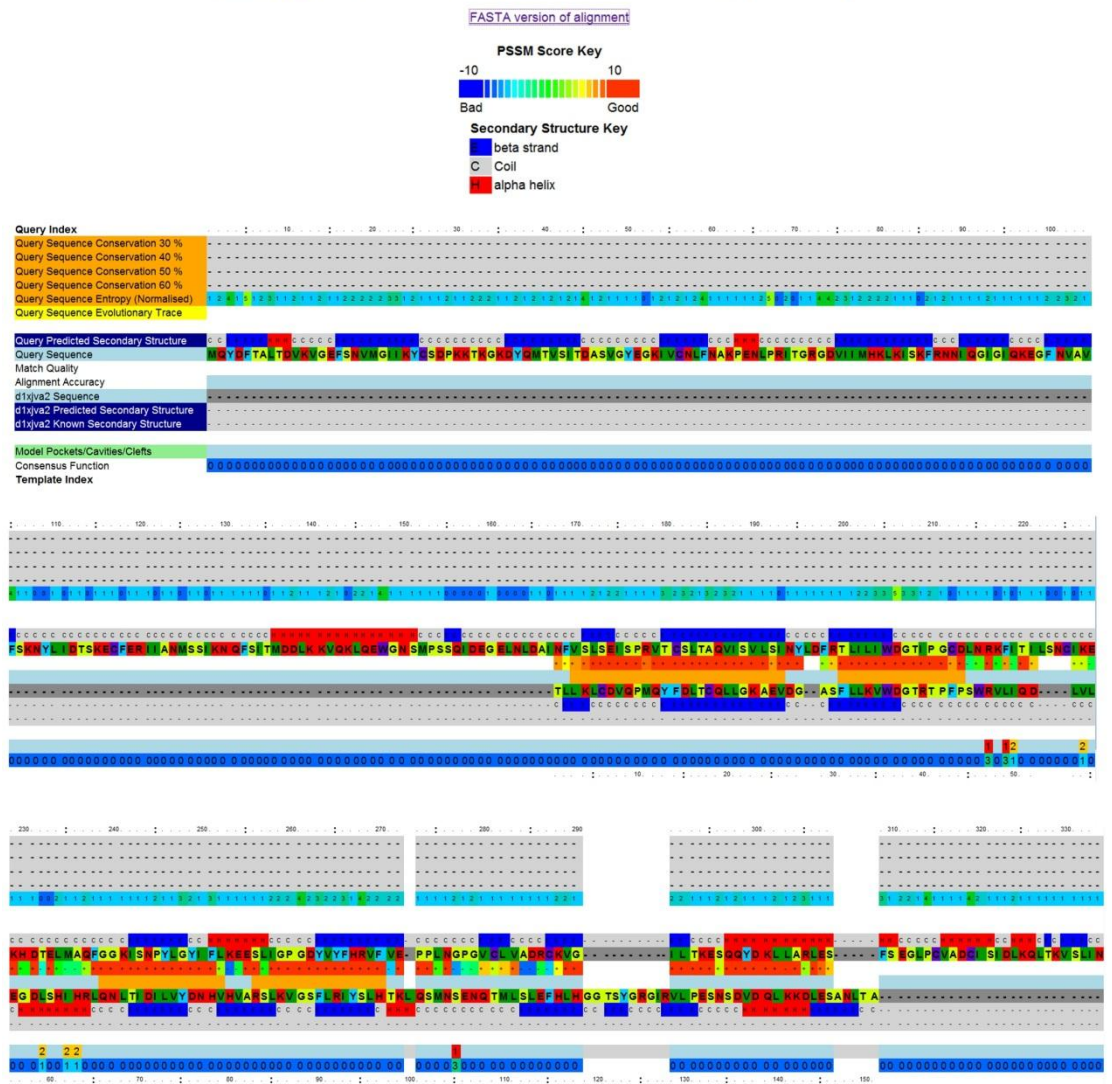
APPENDIX B

POT1 OB1 and OB2 domain alignment



**Figure B1 – Mapping of OB1 fold by homology.** The N-terminal region of POT1 in *S. mediterranea* contains sequence highly similar to the OB1 fold in human POT1.

# QuickPhyre Alignment of query sequence to fold library protein *d1xjva2*



**Figure B2 – Prediction of OB2 fold by Phyre, showing consensus structures shared between the human POT1 OB2 and POT1 in *S. mediterranea* ( $e = 4.4 \times 10^{-7}$ ).**

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